

## Serotyping Scheme for *Campylobacter jejuni* and *Campylobacter coli* Based on Direct Agglutination of Heat-Stable Antigens

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**Campylobacter is now the most frequently reported cause of gastrointestinal disease in England and Wales, yet few isolates are characterized beyond the genus level. The majority of isolates are *Campylobacter jejuni* (90%), with most of the remainder being *Campylobacter coli*. We describe an adaptation of the Penner serotyping scheme in which passive hemagglutination has been replaced by detection of heat-stable antigens by direct bacterial agglutination; absorbed antisera are used where appropriate. This scheme has been used to type 2,407 *C. jejuni* samples and 182 *C. coli* samples isolated in Wales between April 1996 and March 1997. Forty-seven *C. jejuni* serotypes were identified, with the 10 most prevalent serotypes accounting for 53% of the isolates tested; 19% of the isolates were untypeable. Only fifteen *C. coli* serotypes were identified, with three serotypes accounting for 69% of the isolates. This scheme provides a baseline for epidemiological studies of *C. jejuni* and *C. coli*.**

The role of campylobacter as a cause of enteric disease in humans was not fully recognized until the development of isolation methods and selective media during the 1970s (10). Subsequently, the number of human campylobacter infections reported in England and Wales has increased annually, and since 1981 campylobacter has been the most commonly reported cause of acute bacterial enteritis in England and Wales (reports to the Public Health Laboratory Service [PHLS] Communicable Disease Surveillance Centre). Although the organisms in the majority of the 43,240 reports in 1996 were identified simply as "campylobacter", the available data suggest that circa 90% are *Campylobacter jejuni*, 10% are *Campylobacter coli*, and less than 1% are *Campylobacter lari* (10).

A national case-control study in England and Wales during 1990 and 1991 and the interim report of the Department of Health Advisory Committee on the Microbiological Safety of Food (2, 3) recognized that reference subtyping was needed in order to reach a better understanding of both sources of infection and routes of transmission.

There have been a number of studies comparing different methods for subspecies typing within *C. jejuni* and *C. coli*, and both Patton et al. (22) in the United States and Owen and Gibson (18) in the United Kingdom concluded that, for surveillance on a broad scale, serotyping is the most practical solution. Two serotyping schemes for campylobacter developed in Canada in the 1980s have been widely used, either separately or together (23). The Penner scheme (24) is based on soluble heat-stable antigens, while the Lior scheme (14) detected variation in heat-labile antigens. The Penner scheme has been more widely used in the United Kingdom (13, 27) and was therefore used as the basis for further development by the Laboratory of Enteric Pathogens (LEP).

Two main drawbacks of the Penner scheme have been identified (18). Passive hemagglutination (PHA) was introduced as the detection system in an attempt to eliminate nonspecific agglutination reactions. However, reproducibility problems

can occur as a result of variation in the source, age, concentration, and condition of the erythrocytes (8, 18). Also, the Penner scheme used unabsorbed antisera and a significant proportion of isolates agglutinated more than one antiserum, particularly serogroups 4, 13, 16, and 50 (13) or 4, 13, 16, 43, and 50 (21).

The present paper describes a modified serotyping scheme for *C. jejuni* and *C. coli* based on the use of absorbed antisera to heat-stable antigens and utilizing whole-cell agglutination to replace PHA.

### MATERIALS AND METHODS

**Bacterial isolates.** Sixty-six type strains for the Penner campylobacter serotyping scheme, 47 *C. jejuni* strains and 19 *C. coli* strains, were obtained from the National Collection of Type Cultures (NCTC; PHLS, London, United Kingdom). Antisera raised against these strains have been used to type 2,407 *C. jejuni* samples and 182 *C. coli* samples isolated as part of a pilot study for the PHLS Campylobacter Reference facility carried out between April 1996 and March 1997 in conjunction with the PHLS and National Health Service hospital laboratories in Wales. All isolates are stored at  $-80^{\circ}\text{C}$  in cryovials (Microbank; Pro Lab Diagnostics, Richmond Hill, Ontario, Canada).

**Culture, identification, and determination of species.** Campylobacter isolates were cultured on Columbia blood agar (Oxoid CM331; Unipath, Basingstoke, United Kingdom) with 5% horse blood at  $37^{\circ}\text{C}$  in a variable-atmosphere incubator (VAIN; Don Whitley Scientific Ltd., Shipley, West Yorkshire, United Kingdom) under microaerobic conditions (5%  $\text{CO}_2$ , 5%  $\text{O}_2$ , 3%  $\text{H}_2$ , and 87%  $\text{N}_2$ ). Identification was confirmed by testing for microaerobic growth at 25 and  $42^{\circ}\text{C}$ , oxidase and catalase production, and indoxyl acetate hydrolysis. *C. jejuni* and *C. coli* were differentiated on the basis of hippurate hydrolysis as described by Bolton et al. (6).

**Antiserum production.** Antisera were prepared in New Zealand White rabbits against the strains shown in Table 1. Bacterial suspensions in saline were standardized ( $10^9$  cells/ml) and heated at  $100^{\circ}\text{C}$  for 30 min. Preimmune antisera were prepared from blood samples taken via the marginal ear vein before immunization. The rabbits received 0.5 ml of bacterial suspension intravenously on day 1 followed by 1 ml on days 5 and 10 and 2 ml on days 15 and 20. Antibody levels were assessed in antiserum samples from approximately 5 ml of blood from a marginal ear vein taken on day 25. If the titer was 320 or greater, the rabbits were bled and antisera were separated and stored at  $-20^{\circ}\text{C}$ . Where antibody levels were inadequate, two further injections of 2 ml each were given at 5-day intervals and the rabbits were bled on day 40.

**Absorption of typing antisera.** All antisera were titrated against the complete set of type strains. Where a current clinical isolate agglutinated with more than one antiserum, absorptions were performed, essentially as described by Abbott et al. (1). For every milliliter of antiserum, one 9-cm-diameter plate of confluent growth of the cross-reacting type strain was suspended in phosphate-buffered saline (PBS), heated to  $100^{\circ}\text{C}$  for 30 min, and added to the antiserum. After being mixed the mixture was incubated for 2.5 h at  $50^{\circ}\text{C}$  before centrifugation to

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remove cells from the absorbed antiserum. This absorbed antiserum was then tested against all type strains which reacted with the unabsorbed antiserum. Absorptions were repeated until cross-reactions no longer occurred. All resulting monospecific antisera were diluted to 1:40 for routine use.

**Serotyping by direct agglutination.** Growth harvested from a 24-h plate culture was resuspended in 1 ml of PBS to produce a dense suspension; this was heated at 100°C for 30 min. A standard dilution was made by adding the boiled suspension to 5 ml of PBS to an opacity equivalent to McFarland standard 4 (API, Vercieu, France). This suspension is stable at 4°C for at least 20 weeks. For serotyping, 25- $\mu$ l aliquots of a 1:40 dilution of the reference antisera were dispensed into a U-bottom-well microtiter tray and 25  $\mu$ l of test suspension was added. The tray was incubated at 50°C for 2.5 h in a moist atmosphere with gentle agitation on an orbital shaker. Agglutination was read at 1.5 h, and negative isolates were reincubated for a further 1 h. It was essential that agglutination be read within 30 min of removal of the tray from the incubator. Where agglutination was observed with more than one antiserum, the reacting antisera were titrated against the strain and titers of greater than 40 were regarded as positive.

## RESULTS

**Antiserum production and absorption.** Antisera were produced from all 66 Penner type strains (Table 1) with homologous titers, as measured by direct agglutination, varying from 80 to 2,560. Heterologous agglutination was seen with 47 of the 66 antisera (72.7%). The patterns of cross-reactions observed with the type strains did not necessarily match those observed with current clinical isolates, and an absorption strategy was therefore developed to eliminate the observed cross-reactions occurring with current clinical isolates.

Eighteen antisera gave monospecific reactions with both the type strains and the current clinical isolates. These antisera are used in the serotyping panel unabsorbed, as are a further nine antisera which gave cross-reactions with one or more type strains but not with current clinical isolates. Thirty antisera showed cross-reactions with both type strains and current clinical isolates, and these have been absorbed with one or more of the type strains listed in Table 1.

Three pairs and one trio of antisera gave the same results by the direct agglutination method. All current clinical isolates reacted with both antisera of the pairs or all three antisera of the trio, and absorption with any of the type strains in that group removed agglutination with all of the appropriate antisera. Only one of each of these four groups of antisera is therefore included in the panel of 61 antisera which constitute the routine serotyping panel, and the earlier of the type designations has been conserved (e.g., HS9 has been retained, and HS38 has been discarded).

**Typing of current clinical isolates.** The distribution of serotypes among 2,407 *C. jejuni* samples and 182 *C. coli* samples isolated in Wales between April 1996 and March 1997 is given in Table 2. Forty-seven serotypes were identified for *C. jejuni* (Table 2); for 20 of these, the numbers of samples were more than 1% of the total. For *C. coli* (Table 2), 15 serotypes were identified, six of which were represented by a single isolate. Four hundred and eighty-nine isolates were untypeable (18.9%), and the majority of these failed to agglutinate with any of the antisera used. Seventeen isolates reacted with two or more antisera and are reported as untypeable pending resolution of these cross-reactions. Ten of the 17 reacted with two or more of antiHS13, antiHS16, and antiHS29, i.e., the so-called "HS4 complex" (19).

The proportion of untypeable isolates will be reduced by raising antisera against untypeable isolates to define new types. For example, an antiserum raised against strain C001975 was tested against all other untypeable isolates in this study and against the type strains; five *C. jejuni* isolates agglutinated with anti-C001975. None of the type strains agglutinated with anti-

C001975, and C001975 has therefore been adopted as the type strain for serotype HS67, which has been added to the scheme.

## DISCUSSION

Antisera were produced in rabbits against all 66 Penner serotyping type strains deposited in NCTC and used to develop a serotyping scheme for *C. jejuni* and *C. coli*, based on direct bacterial agglutination. Forty-seven antisera gave cross-reactions with the type strains and, where cross-reactions have also been observed among current clinical isolates, these have been removed by using agglutinin absorption against appropriate type strains (Table 1). The titers obtained from antisera raised against the freeze-dried type cultures were generally relatively low, whereas preparation of antisera against current clinical isolates of the same serotypes resulted in higher titers (unpublished data). Where more than one isolate of the same serotype was used to prepare antisera or when a number of rabbits were injected with the same vaccine, considerable variations in titer were observed (unpublished data).

The LEP serotyping scheme for *C. jejuni* and *C. coli* has addressed the two principal limitations of the Penner serotyping scheme for campylobacters. Reproducibility problems due to variations in erythrocytes (18) have been eliminated by adopting a direct bacterial agglutination method similar to that used for serogrouping schemes for other enteric pathogens. The nonspecific reactions observed in the original serotyping studies of *Vibrio fetus* (5) have been eliminated by incubating the reaction mixtures at 50°C with gentle shaking and reading the result immediately on removal from the shaker. The use of unabsorbed antisera in the Penner scheme resulted in a high proportion of isolates reacting with more than one antiserum (26), and these cross-reactions varied in expression (16). This problem has been addressed by using absorbed antisera to eliminate the cross-reactions observed among current clinical isolates. This has resulted in a scheme which defines 44 heat-stable serotypes for *C. jejuni* and 17 for *C. coli*.

The proportion of untypeable isolates is unsatisfactorily high at 19%. However, this level of typeability is comparable to that from a recent study of poultry campylobacter isolates in The Netherlands where 18% of isolates were found to be untypeable by using the Penner serotyping technique (11). The most recently published data from studies using the Penner scheme for isolates from humans have been those from studies carried out in China and Japan (17) and Ethiopia (4). The quoted untypeability rates are 58.8% for Chinese isolates, 13.0% for Japanese isolates, and 63.4% for Ethiopian isolates. These observations no doubt reflect wide geographical variations in the distribution of *C. jejuni* serotypes. The type strains are representative of campylobacter strains prevalent in the early 1980s, and the majority were isolated in Canada. Antisera raised against untypeable isolates from the present study will be used to extend the scheme and reduce the proportion of untypeable isolates in the United Kingdom by defining new types. One new type, HS67, has been added to date.

Comparability with the Penner scheme is not exact because of the methodological differences detailed above. Penner antisera were prepared with live vaccines, whereas the method described above uses heat-killed whole-cell vaccines. The original Penner scheme used only unabsorbed antisera; Jones et al. (12, 13) reported the use of absorbed antisera, but almost half of the isolates tested still showed some cross-reactions within serogroups 4, 13, 16, and 50. More recently, Jacobs-Reitman et al. (11) have developed a set of absorbed antisera and have used a formalin-fixed inoculum for rabbit immunization.

The principal difference between the Penner and LEP meth-

TABLE 1. Antisera raised against Penner type strains

Vaccine strain used			Antiserum <sup>a</sup>				
NCTC no.	Species	Penner sero-type <sup>b</sup>	LEP designation	Homologous titer with <sup>c</sup> :		Penner type strain(s) (titer[s]) for which cross-reactions observed	Penner type strain(s) with which antiserum absorbed
				I	II		
12500	<i>C. jejuni</i>	1	antiHS1	160	160	5 (80)	5
12501	<i>C. jejuni</i>	2	antiHS2	640	320	7 (320), 8 (40), 10 (80), 13 (320), 22 (80), 44 (80)	13, 44
12502	<i>C. jejuni</i>	3	antiHS3	160			
12503	<i>C. jejuni</i>	4	antiHS4	160			
12504	<i>C. jejuni</i>	5	antiHS5	160	160	1 (20), 47 (80), 66 (160)	1
12505	<i>C. jejuni</i>	6	antiHS6	640	640	12 (80), 25 (320), 26 (80), 59 (80)	25
12506	<i>C. jejuni</i>	7	antiHS7	320			
12507	<i>C. jejuni</i>	8	antiHS8	640	320	17 (160), 44 (40), 47 (80), 53 (80)	17
12508	<i>C. jejuni</i>	9	NI <sup>d</sup>	160		38 (80)	
12509	<i>C. jejuni</i>	10	antiHS10	160	160	50 (40), 65 (80)	50
12510	<i>C. jejuni</i>	11	NI	160			
12511	<i>C. jejuni</i>	12	antiHS12	1280	1280	6 (40), 25 (320), 26 (320), 37 (80), 59 (160)	25
12512	<i>C. jejuni</i>	13	antiHS13	1280	80	4 (640), 7 (160), 16 (640), 19 (640), 22 (320), 29 (320)	29, 50
12526	<i>C. coli</i>	14	antiHS14	320	160	46 (640), 47 (640), 49 (640), 52 (320)	46, 49
12513	<i>C. jejuni</i>	15	antiHS15	640	640	18 (80)	18
12514	<i>C. jejuni</i>	16	antiHS16	1280	1280	2 (160), 4 (160), 10 (80), 13 (320), 19 (320), 22 (160), 29 (80), 46 (320), 50 (80), 62 (80), 65 (40)	13, 29
12515	<i>C. jejuni</i>	17	antiHS17	320			
12516	<i>C. jejuni</i>	18	antiHS18	320			
12517	<i>C. jejuni</i>	19	antiHS19	80			
12527	<i>C. coli</i>	20	antiHS20	320			
12518	<i>C. jejuni</i>	21	antiHS21	640			
12519	<i>C. jejuni</i>	22	antiHS22	320	160	23 (80), 35 (160)	23
12520	<i>C. jejuni</i>	23	antiHS23	320	160	22 (320)	22
12528	<i>C. coli</i>	24	antiHS24	160	160	25 (80), 26 (640)	25
12529	<i>C. coli</i>	25	antiHS25	1280	160	6 (640), 12 (640), 26 (640), 59 (160)	6
12530	<i>C. coli</i>	26	antiHS26	640	320	24 (80), 25 (160)	24
12521	<i>C. jejuni</i>	27	antiHS27	640			
12531	<i>C. coli</i>	28	antiHS28	640		30 (80), 34 (160)	ANR <sup>e</sup>
12522	<i>C. jejuni</i>	29	antiHS29	640	320	7 (40), 50 (320), 65 (320)	50
12532	<i>C. coli</i>	30	antiHS30	320	80	3 (160), 28 (640), 34 (320)	3, 28
12523	<i>C. jejuni</i>	31	antiHS31	320	160	11 (320), 17 (80), 64 (40)	64
12524	<i>C. jejuni</i>	32	antiHS32	160		36 (160)	ANR
12537	<i>C. jejuni</i>	33	antiHS33	320			
12533	<i>C. coli</i>	34	antiHS34	640	640	28 (20), 30 (160)	28
12538	<i>C. jejuni</i>	35	antiHS35	1280	160	22 (160), 29 (640)	29
12547	<i>C. jejuni</i>	36	antiHS36	160	80	32 (160)	32
12539	<i>C. jejuni</i>	37	antiHS37	640	160	12 (160), 25 (160)	12
12540	<i>C. jejuni</i>	38	antiHS9	320	160	63 (320)	63
12534	<i>C. coli</i>	39	antiHS39	320	80	51 (160), 61 (160), 66 (160)	61
12541	<i>C. jejuni</i>	40	antiHS40	640		45 (80)	ANR
12542	<i>C. jejuni</i>	41	antiHS41	160			
12543	<i>C. jejuni</i>	42	antiHS42	160			
12548	<i>C. jejuni</i>	43	antiHS43	160	80	2 (80), 4 (80), 10 (80), 13 (80), 22 (160), 29 (640), 31 (80), 44 (80), 46 (80)	44
12549	<i>C. jejuni</i>	44	antiHS44	320	320	2 (80), 4 (320), 10 (160), 13 (320), 19 (80), 22 (80), 29 (80), 31 (80), 46 (320), 47 (160)	43
12544	<i>C. jejuni</i>	45	antiHS45	640		40 (80)	
12569	<i>C. coli</i>	46	antiHS46	640	640	14 (80), 47 (640), 49 (80), 54 (160)	14
12535	<i>C. coli</i>	47	NI	640		14 (320), 46 (640), 47 (640), 49 (320), 54 (320)	
12536	<i>C. coli</i>	48	antiHS48	1280		7 (320)	ANR
12570	<i>C. coli</i>	49	antiHS49	640			
12559	<i>C. jejuni</i>	50	antiHS50	640	320	16 (80), 65 (640)	16
12550	<i>C. coli</i>	51	antiHS51	160			
12545	<i>C. jejuni</i>	52	antiHS52	160			
12560	<i>C. jejuni</i>	53	antiHS53	160			
12551	<i>C. coli</i>	54	NI	640		14 (320), 26 (80), 46 (640), 47 (640), 49 (320)	
12546	<i>C. jejuni</i>	55	antiHS55	80			
12567	<i>C. coli</i>	56	antiHS56	320		37 (40)	ANR
12552	<i>C. jejuni</i>	57	antiHS57	160		3 (160), 60 (80)	ANR
12553	<i>C. jejuni</i>	58	antiHS58	160	160	11 (640), 17 (160), 31 (320), 64 (160)	64
12568	<i>C. coli</i>	59	antiHS59	80	80	25 (320), 26 (320), 64 (80)	25
12554	<i>C. jejuni</i>	60	antiHS60	320		7 (320)	ANR
12570	<i>C. coli</i>	61	antiHS61	160	160	39 (80), 51 (160), 66 (80)	66
12555	<i>C. jejuni</i>	62	antiHS62	80			
12556	<i>C. jejuni</i>	63	antiHS63	640		9 (40), 38 (640)	ANR
12557	<i>C. jejuni</i>	64	antiHS11	320		11 (640), 17 (160), 31 (320)	ANR
12558	<i>C. jejuni</i>	65	NI	640		16 (40), 50 (320)	
12525	<i>C. coli</i>	5	antiHS66	320	160	5 (160), 39 (160), 51 (160), 61 (160)	39

<sup>a</sup> Titers were measured by the direct agglutination technique.

<sup>b</sup> Penner serotype as given in the NCTC catalog. For Penner serotype 5, strain 12504 is hippurate positive and strain 12525 is hippurate negative.

<sup>c</sup> I, unabsorbed antiserum; II, absorbed antiserum if appropriate.

<sup>d</sup> NI, type not included in the LEP scheme because antisera were indistinguishable by direct agglutination: antiHS9 = antiHS38, antiHS11 = antiHS64, antiHS47 and antiHS54 = antiHS14, and antiHS65 = antiHS50.

<sup>e</sup> ANR, absorption not required for current clinical isolates; see text.

TABLE 2. Serotypes of *C. jejuni* and *C. coli* isolated in Wales between April 1996 and March 1997

Serotype	No. of isolates	% of total
<i>C. jejuni</i>		
HS50	245	10.2
HS2	237	9.8
HS44	183	7.6
HS11	161	6.7
HS16	98	4.1
HS13	95	3.9
HS6	82	3.4
HS37	81	3.4
HS21	71	2.9
HS5	70	2.9
HS1	61	2.5
HS14	56	2.3
HS8	46	1.9
HS18	46	1.9
HS12	41	1.7
HS9	40	1.7
HS35	37	1.5
HS59	34	1.4
HS15	33	1.4
HS29	31	1.3
HS3	21	0.9
HS57	21	0.9
HS60	18	0.7
HS43	17	0.7
HS10	13	0.5
HS27	10	0.4
HS42	7	0.3
HS62	6	0.2
HS7	4	0.2
HS33	4	0.2
HS52	4	0.2
HS38	3	0.1
HS45	3	0.1
HS23	2	0.1
HS41	2	0.1
HS53	2	0.1
HS55	2	0.1
HS63	2	0.1
HS30	2	0.1
HS4	1	0.04
HS22	1	0.04
HS46	1	0.04
HS48	1	0.04
HS49	1	0.04
HS56	1	0.04
HS58	1	0.04
HS61	1	0.04
Untypeable	467	19.4
Rough	41	1.7
Total	2407	
<i>C. coli</i>		
HS56	69	37.9
HS66	33	18.7
HS28	23	12.6
HS14	6	3.3
HS61	5	2.7
HS48	4	2.2
HS6	3	1.6
HS49	2	1.0
HS46	2	1.0
HS25	1	0.5
HS30	1	0.5
HS31	1	0.5
HS35	1	0.5
HS39	1	0.5
HS59	1	0.5
Untypeable	22	12.1
Total	182	

ods lies in the detection system. Whereas the LEP protocol uses direct bacterial agglutination, the Penner method uses PHA, that is, the supernatant from a boiled cell suspension is used to sensitize erythrocytes, which are in turn mixed with antisera in order to demonstrate agglutination. Although the PHA method will be most efficient for soluble antigens, it had been assumed that the *C. jejuni* heat-stable antigens were lipopolysaccharides (LPS) (24). While long-chain LPS have been detected in some serotypes of *C. jejuni* (25), only LPS core and short-chain polysaccharides have been detected in other studies (15), and it has been suggested that the antigen detected by PHA and direct agglutination is capsular (7).

Many workers have noted cross-reactions in isolates belonging to serotypes 4, 13, 16, 43, and 50 (11, 20, 26). These serotypes have sometimes been referred to collectively as the HS4 complex (19). By using the LEP protocol and unabsorbed antisera, cross-reactivity between serotypes 4, 13, 43, 50, and 65 was observed. However, serotypes HS4, HS13, HS16, HS43, and HS50 could be distinguished with absorbed antisera. Only one isolate in this study, other than the type strain, belonged to HS4. Serotypes HS50 and HS65 were indistinguishable by direct agglutination and reciprocal absorption, so HS65 has been dropped from the LEP scheme. A number of clinical isolates which react with both antiHS13 and antiHS16 but which are not resolved by absorption remain, and further studies are in progress to determine the antigenic structures of these isolates. These observations fit with a numerical analysis of pulsed-field patterns carried out to determine lineages within *C. jejuni* (9); this analysis identified three clonal lines within the HS4 complex, namely, HS4/HS13/HS16, HS43, and HS50/HS65.

The combination of absorbed antisera with a direct whole-cell agglutination technique has produced a method which gives a serotyping result in 2 h. The use of a microtiter agglutination technique maximizes the number of tests from each batch of antiserum and lends itself to automation. In order to investigate the epidemiology of an organism when there is a need to type large numbers of sporadic isolates, the chosen typing technique must lend itself to routine use on large numbers of isolates, produce easily interpreted data, and be capable of standardization across a number of users. Serotyping fulfills these requirements, and the LEP scheme for *C. jejuni* and *C. coli* has demonstrated its applicability in this pilot study in Wales. The scheme has now been adopted as the basis of reference typing in England and Wales.

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