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Detection of Heat-Stable Antigens of *Campylobacter jejuni* and *C. coli* by Direct Agglutination and Passive Hemagglutination

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The two serotyping schemes for the detection of heat-stable antigens of *Campylobacter jejuni* and *Campylobacter coli* use the same strains for antiserum production but differ in the detection systems used for identifying agglutination. The Penner method uses passive hemagglutination (PHA) while the Laboratory of Enteric Pathogens method uses the same antisera but in a whole-bacterial-cell direct agglutination (DA) protocol. *C. jejuni* produces a polysaccharide capsule, which is antigenic, and is the main component detected by the PHA method. The DA method will detect both capsule antigens and lipopolysaccharide (LPS) or lipooligosaccharide (LOS) surface antigens. Comparison of both methods by using a selection of isolates from human infection has shown a range of variation in agglutination specificity, reflecting the differences in antigens detected by the two methods. While 27.4% of the 416 *C. jejuni* isolates reacted with the antisera raised against the same type strains by either method, the majority showed a range of more complex relationships. None of the 37 *C. coli* isolates reacted with the same antiserum by both methods. Together the two schemes gave a total of 102 distinct combined serogroups for *C. jejuni* and 16 for *C. coli*. Thus, while some clonally related isolates share the same capsule and LOS or LPS antigens, other strains appear to have a common capsule antigen but differ in their LPS or LOS structures or vice versa.

Campylobacters have been the most commonly reported cause of bacterial enteritis in England and Wales since 1981, with 53,858 cases reported in 2000 (http://www.phls.co.uk/facts/Gastro/Campy/campyQua.htm).

For large-scale surveillance of *Campylobacter jejuni* and *Campylobacter coli*, comparative studies of typing methods (22, 31) have suggested a combination of methods with serotyping as the most practical solution. Although several distinct serotyping schemes have been described, that of Penner and Hennessy (27), which was developed in Canada and detects heat-stable antigens, has been used in the United States (26) and by a number of laboratories in Europe (12, 21). A serotyping scheme for heat-labile antigens was also developed in Canada (16), and this has also been used in a number of other laboratories (2).

The Penner serotyping method detects heat-stable antigens by passive hemagglutination (PHA), in which sheep or chicken red blood cells (RBCs) are sensitized with the supernatant of boiled cell suspensions and reacted with unabsorbed antisera. Pooled absorbed antisera have also been used in conjunction with PHA (11). A modification of the Penner scheme that uses direct agglutination (DA) of boiled whole bacterial cells has been described previously (9). The new approach also utilized absorbed antisera to improve discrimination and reproducibility. Since 1996, the DA method has been used for reference serotyping of campylobacters in England and Wales.

It was recognized at the outset that the change in detection method would not give direct comparability between the two methods (9), and this has been confirmed by subsequent studies (18). During the development of the DA method, it became clear that the antigen detected by the Penner method was not a long chain lipopolysaccharide (LPS) as previously assumed but that it was probably capsular in nature (3). The campylobacter genome sequence demonstrated that loci for both LPS or lipooligosaccharide (LOS) and capsule are present (25), and the relationship between capsule expression and Penner serotype has been confirmed by subsequent genetic studies (12). Recent serological studies (19) have shown that it is possible to purify LOS or LPS and extracellular polysaccharide components from heat-treated preparations and that both contribute to PHA reactions. Similarly, the DA method will detect both capsule and LPS or LOS, depending on the absorption strategy used in antiserum production. In order to further explore the relationship between the PHA and DA agglutination techniques and the antigens detected, a panel of 412 *C. jejuni* and 37 *C. coli* clinical isolates from northwestern England was typed and compared by using both techniques.

MATERIALS AND METHODS

Bacterial strains and serotyping. Campylobacter isolates from human clinical specimens referred to the Preston Public Health Laboratory were isolated and identified by using standard techniques (29). They were typed by the PHA method as described by Penner and Hennessy (27) by using locally produced unabsorbed antisera to 43 of the Penner type strains. Isolates were subsequently referred to the Campylobacter Reference Unit and typed by using DA with absorbed antisera raised against the full set of 66 type strains as described by Frost et al. (9). A subset of isolates, including all isolates which were untypeable by PHA with the Preston antisera, was reexamined by both PHA and DA by

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TABLE 1. Comparison of PHA and DA results for all serotypes of *C. jejuni*[a]

DA serotype	No. of isolates detected by PHA as serotype:																																		
	1	2	44	4	13	16	50	5	11	6	19	21	27	35	37	45	3	7	8	9	10	12	15	17	18	23	24	31	42	46	55	UT	Total
1	11	3																														1	15
2		22																														1	23
44	13	15	1		1																											9	39
4				2																													2
13		2		2	4				1																								9
16				2	4	1	1																									1	9
50		2		68	10		2																									3	85
5		1						3																1								2	7
11								6	36																							1	43
6										6																							6
19											6																						6
21												8																					8
27													1											1									2
35					1									2							1											4	8
37															2																	2	4
45																1																	1
7																		1														2	3
8																			1													2	3
9	1				1				1	2							4								1							3	13
12																																5	5
15																						1											1
17																																1	1
18									2									1							1					1		8	13
23				1					2																	1							4
29																					1												1
33									1																								1
42	1																						1						1				3
52				1		1																											2
53																																1	1
55					1		1																								1	1	4
57																1																1	2
60																	1															1	2
62										3																							3
67																					1												1
68																										1						1	2
UT	11	11		2		1	1	5	4		1			1			1					2		1	2	2	4	1	1	1		3	29	84
Total	37	56	1	78	22	3	5	14	47	11	7	8	1	3	2	2	6	2	1	1	4	1	2	4	4	6	1	1	2	1	4	79	416

[a] Boxed values are serogroups which show similar relationships between PHA and DA. UT, untypeable.

using Laboratory of Enteric Pathogens (LEP) antisera. All isolates were stored at −80°C in Microbank cryovials (Prolab PL1605/G).

Campylobacter isolates were cultured on Colombia blood agar (Oxoid CM331; Unipath, Basingstoke, United Kingdom) with 5% horse blood at 37°C in a variable atmosphere incubator (Don Whitley Scientific Ltd., Shipley, United Kingdom) under microaerobic conditions (5% CO_2, 5% O_2, 3% H_2, and 87% N_2).

All serotyping was carried out on freshly isolated cultures or cultures which had been subbed three times on Columbia blood agar following recovery from storage at −80°C. Where agglutination was observed with more than one antiserum, the reacting antisera were titrated in doubling dilutions from 1:10 to 1:640 against the strain and only titers of greater than 1:40 were regarded as positive (9).

For clarity, the designation PHA1 is used hereafter to denote the serotype of isolates which reacted with antiserum to the Penner type 1 strain by the PHA method, and DA1 is used for the serotype of isolates reacting with the antiserum by the DA method. Thus, the composite serogroup designation will be PHA1: DA1.

Cross-absorption studies. Whole-cell absorption was carried out by incubating 1 ml of heat-killed bacterial cells with 50 μl of the appropriate antiserum at 50°C for 2.5 h before centrifugation (900 × *g*, 10 min) followed by filtration (0.2-μm pores; Sartorius, Göttingen, Germany) of the supernatant. For RBC absorption, chicken RBCs were sensitized as for PHA typing. Absorption was carried out by incubating 1 ml of sensitized RBCs with 50 μl of the appropriate antiserum at 37°C for 1 h before centrifugation and followed by filtration of the supernatant as above. After a final centrifugation (11,000 × *g*, 5 min) of the absorbed antisera, agglutination tests were carried out as described for serotyping.

Molecular identification. Where a *C. jejuni* isolate reacted with antiserum raised against *C. coli*, or vice versa, the speciation of the isolate was confirmed by using molecular methods. *C. jejuni* and *C. coli* are differentiated by the ability to hydrolyze hippurate. *C. jejuni* isolates are hippurate positive, although atypical isolates, which are phenotypically negative but have the hippuricase gene, have been described. Speciation was established by detection of the hippuricase gene by PCR (30) and confirmed by a 23S rRNA PCR restriction fragment length polymorphism method (10).

RESULTS

***C. jejuni*.** A total of 102 distinct serogroups were identified among the 416 *C. jejuni* isolates. One hundred fourteen isolates (27.4%) reacted with the same antiserum by either method while 29 (6%) were untypeable by both methods (Table 1). Isolates which were untypeable by PHA with the Preston antisera were retyped by PHA using the full set of LEP antisera. Untypeability by either technique was 19% for PHA and 20% for DA. Fifty isolates were untypeable by PHA but were typed with the DA method, and 46 isolates were untypeable by DA but were typed by PHA. There were 31 serotypes detectable by PHA and 35 serotypes detectable by DA.

With one exception, all of the *C. jejuni* isolates reacted with

TABLE 2. Agglutination reaction results of PHA21:DA21 isolates reacted with antisera absorbed with homologous whole cells, sensitized RBCs, and controls

Antiserum	Cell type[a]	Agglutination with HS21 by:	
		DA	PHA
Anti-HS18	None[b]	–	–
Anti-HS21	None[b]	+	+
	NCTC12516 (HS18) whole cells	+	+
	Unsensitized RBCs	+	+
	NCTC12518 (HS21) whole cells	–	–
	RBCs sensitized with NCTC12518 (HS21)	+	–

[a] Strains from the National Collection of Type Cultures are the Penner type strains as used for antiserum production (8).
[b] Control reaction.

antisera raised against *C. jejuni* type strains. One isolate typed as PHA46, a *C. coli* type, but DA18. Molecular fingerprinting with 23S rRNA restriction fragment length polymorphism confirmed that this strain was a *C. jejuni* isolate.

A spectrum of PHA:DA serogroups was observed ranging from simple matches, in which there was direct comparability between the two schemes, to a more complex relationship. Table 1 shows the relationships between PHA and DA serotypes. The types have been grouped to demonstrate the range of different patterns seen between the two methods. Strains which were untypeable by one of the two methods have not been included in these groups but are shown at the bottom and right-hand side of Table 1.

Direct correspondence between PHA and DA serotype was shown by isolates of serogroups PHA21:DA21, PHA19:DA19, and other less common serogroups grouped together in Table 1. In these serogroups, the majority of isolates reacted with the same antiserum by either PHA or DA and the results were reproduced whether Preston or LEP antisera were used for PHA.

Further investigations were carried out on at least two strains of serogroups PHA19:DA19 and PHA21:DA21 by using cross-absorption and heated antigen suspensions to determine whether both methods were detecting the same antigen(s). Both serogroups showed the same response, and these experiments are illustrated in Table 2 with respect to PHA21:DA21. Absorption with homologous whole cells predictably eliminated both PHA and DA reactions. The specificity of the agglutination reaction was confirmed by absorption with a heterologous strain. Absorption with untreated chicken RBCs had no effect on either PHA or DA. However, when RBCs were sensitized with the homologous strain, the PHA reaction was eliminated although the DA reaction was unaffected.

The most complex relationship was shown by strains reacting with antisera 1, 2, and 44 (Table 1), which are among the more prevalent types detected in England and Wales by either method. Of the 65 isolates assigned to one of these three serotypes by both methods, 34 (52%) reacted with the same antiserum in both PHA and DA. A further 22 strains were PHA1 or PHA2 but untypeable by DA while 11 were DA1 (1), DA2 (1), or DA44 (9) but untypeable by PHA. PHA1 strains were equally divided between DA1, DA44, and untypeable while PHA2 strains were predominantly DA2, DA44, or untypeable. Conversely, DA44 strains were mostly PHA1 or PHA2. These results, which were confirmed by using the same (LEP) antiserum with both techniques, demonstrated an overlapping distribution of the antigens detected by the two schemes.

The most common serotypes identified by the PHA methods were in the group comprised of PHA4, PHA13, PHA16, and PHA50, accounting for 108 (26.0%) of all *C. jejuni* isolates. These largely corresponded to DA50 (Table 1). Sixty-eight strains typed as PHA4:DA50, 10 typed as PHA13:DA50, and 2 typed as PHA50:DA50.

Less complex relationships are seen among strains reacting with antisera raised against Penner type strain 11 (Table 1). Thirty-six strains matched by both methods, i.e., serogroup PHA11:DA11. Of the seven remaining DA11 strains, six were PHA5 and one remained untypeable by PHA. Seven strains typing as PHA11 were distributed over five DA types, and the remaining four were untypeable by DA.

***C. coli*.** Of the 37 *C. coli* isolates, none gave the same type by both methods (Table 3). Seventeen strains were untypeable by PHA and 16 were untypeable by DA; only 8 strains were untypeable by both methods. By DA, only one of these strains reacted with an antiserum raised against a type strain of *C. jejuni.* These two species are differentiated by hippurate hydrolysis, with *C. jejuni* being hippurate positive. When tested for the presence of the hippuricase gene by PCR (30), one strain originally classified as *C. coli* was found to be an atypical strain of *C. jejuni* which was phenotypically hippurate negative although the hippuricase gene was present. By PHA, 14 *C. coli* strains reacted with sera raised against *C. jejuni* type strains although all were confirmed as hippuricase gene negative by PCR.

DISCUSSION

The two serotyping methods which detect heat-stable antigens both used heated antigen suspensions in which the heat-labile antigens detected by the Lior method are inactivated. While both use antisera raised against the same type strains, the PHA method uses extracted soluble antigens bound to RBCs as the detection method and has conventionally used unabsorbed antisera. This means that many isolates react with more than one antiserum and that variation in expression has proven to be a problem in data interpretation (17). We have found that low titer cross-reactivity and untypeability vary in both methods depending on the history of the isolate, and

TABLE 3. Comparison of PHA and DA results for all serotypes of *C. coli*[a]

DA serotype	No. of isolates detected by PHA as serotype:									
	4	9	11	37	39	44	45	46	UT	Total
28								1	1	2
50	1									1
56		1		3			4		7	15
66			1		1				1	3
UT	1	1		1	2	1		2	8	16
Total	2	2	1	4	3	1	4	3	17	37

[a] UT, untypeable.

vigilant control of culture conditions is essential. The best results are obtained with freshly isolated strains, and isolates express antigen poorly when recovered from storage at low temperatures. For this reason all isolates in the present study were subbed three times following recovery from −80°C before typing. In addition, as in the original study (9), only agglutination reactions at a titer of greater than 1:40 were regarded as positive.

The DA serotyping method was developed from the Penner method with the aim of replacing PHA with whole-cell DA, which is more analogous to the somatic antigen typing of other enteric pathogens, and minimizing cross-reactions by using absorbed antisera (9). Some workers (11) have addressed the problem of cross-reactions by using absorbed antisera for the Penner method, and this has reduced the proportion of cross-reacting isolates. Whole-cell DA detects antigen on the bacterial cell surface, and this difference in detection method results in a lack of concordance between the two methods. Recent evidence with monoclonal antibodies generated against heat-treated preparations of serotypes PHA1, PHA2, PHA3, and PHA4 showed that extracts were individually reactive with either LPS or LOS or extracellular polysaccharide but not with both.

Both immunological (3) and genetic (12) evidence suggests that the antigen responsible for the PHA reaction is capsular in nature and that the capsule antigen binds to the sensitized RBC and is detected by PHA. While DA is also able to detect the capsular polysaccharide, it will also detect LPS or LOS antigen which remains bound to the cell surface. It has been shown that the majority of *C. jejuni* isolates do not express long chain LPS (3; A. Moran, T. U. Kosunen, and M. M. Prendergast, abstr. H35 from the 11th International Workshop on Campylobacter, Helicobacter, and Related Organisms, Int. J. Med. Microbiol. **291**(Suppl. 31)**:**72–73, 2001) but have a low-molecular-weight LOS which is serotype specific and antigenically active (17, 20). DA has been shown by RBC absorption to detect a different antigen from that detected by PHA. Thus, even in those strains which react with the same antiserum by either method, the contribution of capsular and LPS or LOS antigens to the agglutination reaction will depend on the detection method and absorption strategy used.

Recent genetic analysis of the genes involved in LOS biosynthesis has shown that there can be considerable variation within a single Penner serotype (M. Gilbert, A. van Belkum, W. W. Wakarchuk, E. Taboada, C. W. Antigen, N. van der Braak, C. M. Szymanski, M. F. Karwaski, B. C. Jacobs, J. H. Nash, P. C. R. Godschalk, and P. Endtz, abstr. N31 from the 11th International Workshop on Campylobacter, Helicobacter, and Related Organisms, Int. J. Med. Microbiol. **291**(Suppl. 31)**:**128, 2001). Gilbert and colleagues compared the sequenced strain NCTC 11168 with GB11, an isolate from a Guillain-Barré syndrome patient. Both belong to PHA2 and Lior heat-labile serotype 4 and clustered together when examined by amplified fragment length polymorphism (AFLP). It was observed that the LOS biosynthesis genes of GB11 were almost identical to those of the PHA19 type strain but showed significant divergence from NCTC11168.

That a proportion of isolates reacted with the same antiserum in both detection methods indicates the clonal nature of these serogroups. Genotypic studies of serogroups PHA21:DA21 and PHA19:DA19 with either multilocus sequence typing (MLST) (5) or fluorescent AFLP (4) have demonstrated the strongly clonal nature of these groups compared to the looser relationship within some of the other serogroups. Serogroup PHA19:DA19 has been associated with Guillain-Barré syndrome (20) and other neurological complications following campylobacter infection. Comparisons of pulsed-field gel electrophoresis profiles for isolates from North America, the Far East (7), and England and Wales (14) demonstrated the stability and lack of variation within the PHA19:DA19 clone over a significant time span and wide geographical distribution.

At the other end of the spectrum, particularly in the complex group including serogroups 1, 2, and 44 and the 4-complex, a range of serogroup combinations is observed. Indeed, the two methods could be used together to increase strain discrimination. These serogroups also show considerable variation and complex interactions with other subtyping methods, including phage typing (8), pulsed-field gel electrophoresis (24), fluorescent AFLP (4, 6), and MLST (5), indicating a more panmictic phylogeny. Gene sequence data suggest that the variation within these groups reflects a high rate of recombination (5). This undoubtedly constitutes a survival strategy for campylobacters, enabling the organism to adapt rapidly to a wide range of host environments. Despite this plasticity, it is clear that the serogroups which predominate now are the same as those which were most common in Canada when the Penner serotyping scheme was originally devised (27, 28).

Combined typing strategies (8, 22, 23) have yielded a level of fine discrimination for epidemiological study and shown a wide range of variation in *C. jejuni*, although some prevalent clonal subtypes can still be clearly identified. The combination of two serotyping methods described here detects a similar range of diversity. Furthermore, as the two separate antigens can be detected using the same set of antisera, this level of discrimination could be achieved with only one set of antisera and a common source of antigen suspension.

The lack of concordance between the two methods seen with *C. coli* suggests that the relationship between the surface and capsule antigens is different in this species. Using the DA method, all *C. coli* isolates that are typeable react only with antisera raised against *C. coli* type strains. This suggests that the cell surface antigen detected by DA is more species specific than the capsule. For general use, serotyping of *C. coli* by either the PHA or DA method would require specific further development. For studies of human infection, where *C. coli* accounts for less than 10% of isolates, this is not as critical as it would be for veterinary studies. The use of molecular techniques identified a number of anomalies between strains identified phenotypically as *C. coli*, by using hippurate hydrolysis, and the presence of the hippuricase gene as detected by PCR. This study emphasizes the caution which must be exercised when using a single phenotypic identification or typing test to characterize isolates of *Campylobacter* spp.

The availability of sequence-based typing methodologies, such as an MLST-like approach or the use of DNA microarrays, together with the data on target sequences emerging from postgenomic studies on campylobacter means that the basis for a molecular serotyping method is becoming available. This could employ sequences specific for both capsule and LOS or LPS structural genes. Clarification of the nature and role of the

two heat-stable antigens described here will be an essential component of such developments.

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