Typing of Heat-Stable and Heat-Labile Antigens of *Campylobacter jejuni* and *Campylobacter coli* by Coagglutination

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A coagglutination system has been devised for typing heat-stable and heat-labile antigens of *Campylobacter jejuni* and *C. coli*. The use of protein A-positive *Staphylococcus aureus* cells carrying *Campylobacter* sp. serotype antibody and the treatment of *Campylobacter* sp. cells with DNase in the antigen suspension permitted rapid and specific coagglutination of rough (autoagglutinable) as well as smooth cultures. Cells of *S. aureus* were sensitized with *Campylobacter* sp. serotype antisera. Four to five types of sensitized *S. aureus* cells were pooled.

A strain of *Campylobacter* sp. was first tested with the pools and then typed with the individual reagents of the reactive pool. After the described procedures, 68 serotype strains tested blindly as unknowns were correctly typed according to their heat-stable or heat-labile antigens. The two most commonly used typing schemes which are based separately on the heat-stable or the heat-labile antigens as assayed by passive hemagglutination and slide agglutination, respectively, can be utilized simultaneously in the coagglutination system for strain characterization.

The coagglutination system is simple, yields results rapidly, conserves typing reagents, and offers the flexibility of formulating the pools of reagents according to the experimental design or the prevalence of serotypes in a geographic location. It should be a practical system for the typing of *Campylobacter* spp. in public health or clinical laboratories.

The recent recognition of *Campylobacter* sp. enteritis as a common bacterial diarrheal disease in humans has caused considerable interest in the serotyping of *Campylobacter* sp. isolates (1, 3, 5, 7). Serotyping has contributed significantly to our understanding of the transmission and pathobiology of *Campylobacter* sp. infection. The two most commonly used typing schemes are based on either the heat-stable antigens by a passive hemagglutination assay (8) or the heat-labile antigens as detected by slide agglutination (6). Serotyping of *Campylobacter* sp., however, has been beyond the scope of many public health and clinical laboratories because of the numerous serotypes and because certain typing procedures are tedious and time-consuming. In this report, we describe a coagglutination system by which *Campylobacter* sp. cultures can be typed by their heat-stable and heat-labile antigens in a single operation. The characteristics of the coagglutination system are discussed.

MATERIALS AND METHODS

Bacteria. Sixty-eight strains of *Campylobacter jejuni* and *C. coli* were used. Fifty-seven strains, representing 57 different serotypes according to their heat-stable antigens as assayed by passive hemagglutination (8), were originally obtained from John Penner of Toronto University, Toronto, Ont., Canada. The strains were assigned the prefix P and designated numerically from P1 to P56 corresponding to their respective serotypes, with one strain as P5+ (hippurate positive) and another strain as P5− (hippurate negative). Eleven strains, representing the 11 most prevalent serotypes in the United States as characterized by their heat-labile antigens with the slide agglutination test (C. M. Patton, unpublished data), were originally from H. Lior of the Laboratory Center for Disease Control, Ottawa, Ont., Canada. These strains were designated with the L prefix as L1, L2, L4, L5, L6, L7, L9, L11, L16, L17, and L36.

The bacteria were grown on Columbia agar supplemented with 5% rabbit blood at 42°C in an atmosphere containing 7% CO₂. A buffered solution was formulated to preserve the bacterial antigens without gumminess. It was composed of phosphate-buffered saline (PBS), pH 7.2, with 0.1% bovine serum albumin (BSA), 0.05% sodium azide, 1 µg of DNase per ml, 0.01% CaCl₂, 0.01% MgCl₂, and 0.002% MnCl₂. After incubation for 24 to 48 h, one-half of the bacterial growth was harvested with a moist cotton swab into the preserving solution. The bacterial suspensions were stored at 4°C and used as unheated antigens. The other half of bacterial growth was suspended in PBS and heated to 100°C for 1 h. The bacteria were sedimented by centrifugation and suspended in the preserving buffer for use as heat-antigen.

When antigens were to be used immediately and preservation was not necessary, heated and unheated cultures were suspended in PBS containing 1 µg of DNase per ml without BSA and other ingredients.

Determination of “smooth” or “rough” strains. To assess the effect of nontypical cell agglutination in the coagglutination test, the pattern of cell settlement was determined for each strain. Cultures were suspended in PBS containing 0.1% BSA and diluted to a concentration of approximately 50% transmission at 560 nm. An aliquot of 100 µl of culture suspension was dispensed into each U-bottomed microtiter well. The cells were allowed to settle without disturbance at 4°C overnight. The pattern of cell settlement was examined under a dissecting microscope. Smooth strains settled at the bottom of the wells as a round button with smooth edge and an appearance of ground glass; rough strains settled as a wrinkled sheet of cells attached to the bottom of the wells with a false appearance of cell agglutination.

Antisera. Antisera were produced in young adult rabbits of the New Zealand strain. Antisera for the 57 types of heat-stable antigens were prepared and tested by the passive hemagglutination assay as described by Penner and Hennessy (8). Antisera for the 11 types of heat-labile antigens were prepared by immunizing rabbits intravenously with
formaldehyde-treated serotype cultures (6). Both unab- 
sorbed and absorbed sera were used in experiments on 
typing heat-labile antigens (see Fig. 1). Absorption of anti-
sera for the monospecific heat-labile antigenic types used in 
this study was performed as described by Lior and co-work-
ners (6).

Typing reagents for coagglutination. A 1% suspension of 
lyophilized cells of Staphylococcus aureus (protein A-posit-
tive Cowan strain; Sigma Chemical Co., St. Louis, Mo.) in 
PBS was prepared by shaking the suspension with glass 
beads, followed by passage of the suspension several times 
from a syringe through a 25-gauge needle. The suspension 
was divided into 5-ml aliquots, and 0.5 to 0.8 ml of a Campylobacter sp. typing serum was added to each aliquot. 
The mixture was incubated for 1 h at room temperature with 
an occasional shaking. The antibody-sensitized cells of S. 
aureus were separated from the supernatant by centrifugation, 
washed twice with PBS, and suspended in 10 ml of PBS 
containing 0.05% sodium azide. The sensitized cells of S. 
aureus were stored as a suspension in glass screw-cap tubes 
at 4°C.

Coagglutination test. Coagglutination was performed by 
mixing 20 μl of Campylobacter sp. cell suspension with an 
equal volume of typing reagent on an agglutination slide with 
ceramic rings. The reactants were mixed thoroughly with a 
wooden applicator, followed by rotation of the slide for 1 
min. Agglutination was examined with a dissection micro-
scope and graded on a scale of 1 to 4+ according to the 
approximate amount of agglutination (1+ = 25%, 2+ = 50%, 
3+ = 75%, 4+ = >75%).

The procedures for typing heat-stable and heat-labile 
antigens are detailed in Fig. 1. A suspension of the unheated 
bacterial culture was first tested with the pools of reagents 
prepared with unabsorbed antisera and then presumptively 
identified by testing it with the individual typing reagents 
making up the reactive pool. A confirmatory test was 
performed with a heated culture for heat-stable antigens and 
with a monospecific typing reagent for heat-labile antigens.

The reagents for typing the heat-stable antigens were 
pooled in two different ways in this study. First, the 57 types 
were divided in numerical order into 11 pools, with the first 
and the last pools consisting of six types and the other 9 
pools consisting of five types each. Second, the heat-stable 
antigenic types were divided into the C. jejuni group and the 
C. coli group according to Penner and co-workers (9), and 
the most prevalent types of each group were pooled as 
follows: C. jejuni—pool AJ, types 1, 2, 3, and 4; pool BJ, 
types 5+, 6, 7, and 8; pool CJ, types 10, 11, 13, and 16; pool 
DJ, types 18, 19, 21, and 23; pool EJ, types 31, 37, 42, and 
50; C. coli—pool FC, types 5−, 14, 20, and 24; pool GC, 
types 25, 26, 28, and 30; pool HC, types 34, 39, 46, and 47; 
pool IC, types 48, 49, and 51.

The 11 most prevalent types of heat-labile antigens in the 
United States were grouped as follows: pool HL1, types L1, 
L2, L4, and L5; pool HL2, types L6, L7, L9, and L11; pool 
HL3, types L16, L17, and L36. For pooling, aliquots of 1 ml 
of the antibody-sensitized S. aureus cells were mixed. Each 
pool was tested with the individual strains of Campylobacter 
sp. for coagglutination before use for typing.

RESULTS

Coagglutination activities. Campylobacter sp. cells coag-
glutinated with cells of S. aureus sensitized with the Cam-
pylobacter sp. typing sera. Coagglutination usually occurred 
within <1 min after the reactants were mixed on a glass 
slide. The speed of coagglutination depended on the titer of 
the typing serum used to sensitized the S. aureus and on the 
nature of the Campylobacter sp. serotype antigen. Campy-
lobacter sp. cells generally coagglutinated more readily in 
typing reagents for heat-labile antigens with the appearance 
of flocculation, probably reflecting that flagella and other 
readily accessible surface antigens were involved in the 
agglutinating activities (6). Some strains of Campylobacter 
sp. coagglutinated more slowly (up to 1 to 2 min after 
mixing) in typing reagents for heat-stable antigens, and the 
cell aggregates appeared more compact and granular, 
consistent with agglutinating characteristics of somatic antigens.

In the coagglutination system, specific agglutination oc-
curred rapidly before nonspecific or autoagglutination of 
some rough strains took place. Coagglutination, therefore, 
was not affected by whether the cultures were "smooth" or 
"rough." Approximately 30% of the cultures used in this 
study were "rough" as determined by their settlement pattern 
in microtiter wells, but all cultures coagglutinated specifically 
within <2 min with their respective typing reagents. The 
cell settlement pattern was particularly relevant to an 
tagglutination test. Although not all "rough" cultures 
autoagglutinated in normal sera, the autoagglutina-
ting strains invariably showed a "rough" settlement pattern.

Typing of cultures. After the individual typing reagents 
and the pools were tested with the respective Campylobac-
ter sp. serotype cultures, the cultures were coded and tested 
randomly as "unknowns." The identity of the cultures 
except their coded numbers was not known to the labora-
tory personnel performing the test. After the procedures 
using unheated and heated cultures as detailed in Fig. 1, all 
"unknown" cultures were correctly typed by their heat-sta-
ble or heat-labile antigens as defined by Penner and Henn-
essay (8) and Lior and co-workers (6), respectively. The 
results are summarized in Table 1.

We observed significantly fewer cross-reactivities among 
different heat-stable antigenic types in the coagglutination 
than in the passive hemagglutination test. When cross-reac-
tions occurred, the homologous typing reagents always gave 
stronger and faster coagglutinating activities. Results on 
some of the more cross-reactive strains are summarized in 
Table 2.

After the "unknowns" were identified by coagglutination 
for their heat-stable or heat-labile antigenic types, cultures 
were randomly selected from the heat-stable antigenic types 
(cultures with the P prefix) and from the heat-labile antigenic 
types (cultures with the L prefix) for cross-typing. The 
pools from AJ to IC were used to identify heat-stable antigenic 
types of the L-prefixed cultures, and the HL1 to HL3 pools 
were used to determine the heat-labile antigenic types of the 
P-prefixed cultures. The typing results by coagglutination 
were confirmed by passive hemagglutination for heat-stable

<table>
<thead>
<tr>
<th>TABLE 1. Typing of C. jejuni and C. coli by coagglutination</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of serotypes</td>
</tr>
<tr>
<td>-------------------</td>
</tr>
<tr>
<td>Heat-stable antigens*</td>
</tr>
<tr>
<td>57 (used in pooling by numerical order)</td>
</tr>
<tr>
<td>35 (used in pooling by species and prevalence)</td>
</tr>
<tr>
<td>Heat-labile antigens*</td>
</tr>
<tr>
<td>11 (used in pooling by prevalence)</td>
</tr>
</tbody>
</table>

a Antigenic types determined by coagglutination were confirmed by passive hemagglutination.

b Results by coagglutination were confirmed by slide agglutination.
A. Typing of heat-stable antigens

Step 1. Identification of reactive pool:
A drop of UNHEATED test culture is mixed with each pool of typing reagents.
(See footnote)

Step 2. Presumptive identification of serotype:
A drop of UNHEATED test culture is mixed with each typing reagent constituting the reactive pool.

Culture presumptively identified as serotype 6

Step 3. Confirmation of serotype:
A drop of HEATED test culture is mixed with the reactive typing reagent.

Test result invalid.

FIG. 1. Schema for typing heat-stable (A) and heat-labile (B) antigens of C. jejuni and C. coli by coagglutination. (A) Typing reagents are protein A-positive S. aureus cells sensitized with the respective unabsorbed antisera from rabbits immunized with live serotype cultures. (B) Typing reagents used in steps 1 and 2 are protein A-positive S. aureus cells sensitized with the respective unabsorbed antisera from rabbits immunized with formaldehyde-treated serotypic cultures. Monospecific typing reagent prepared with absorbed antiserum of the reactive serotype is used in step 3 for confirmation. ®, Reagent prepared with unabsorbed antiserum; ○, monospecific reagent.
antigenic types and by slide agglutination for heat-labile antigens. Representative results are given in Table 3. It is evident that *Campylobacter* sp. strains could be simultaneously typed for their heat-stable as well as heat-labile antigens by the simple coagglutination system with a batch of well-defined typing reagents.

**Quality of typing reagents.** After the procedures given in Fig. 1, we found that the unabsorbed immune rabbit sera prepared by the method of Penner and Hennessy (8) were adequate for typing the heat-stable antigens by coagglutination. Since it is tedious and time-consuming to prepare monospecific typing antisera by absorption as described by
Lior and co-workers for the slide agglutination test (6), the coagglutination procedure in Fig. 1 for identifying heat-labile antigens was designed to conserve the consumption of absorbed sera by use of unabsorbed immune sera in the presumptive test. The result was then confirmed with an aliquot of 20 μl of the *S. aureus* cells sensitized with an absorbed monospecific antiserum.

For sensitizing *S. aureus* cells, we obtained strong coagglutination when the passive hemagglutinating titers for the heat-stable antigens were 5,120 or higher and the slide agglutinating titers for the heat-labile antigens were 320 to 640 or higher. When stored in glass screw-cap tubes at 4°C with 0.05% sodium azide as preservative, the sensitized *S. aureus* reagents remained stable without decrease in coagglutinating activity for at least 8 months, the longest period tested.

The suspensions of *Campylobacter* sp. cells used as antigens were stable at 4°C for at least 6 months in the presence of BSA and DNase. Gumminess was not observed. Gram stain revealed many deformed coccolid and blebby cells in the unheated cultures after prolonged storage, but the morphology did not appear to affect the coagglutinating activity or its specificity as identical typing results were obtained throughout the study period of at least 6 months.

**DISCUSSION**

The coagglutination procedure has several advantages that may help to overcome some major drawbacks of the currently used typing methods and extend the capability of typing *C. jejuni* and *C. coli* beyond specialty laboratories.

The use of protein A-positive *S. aureus* as a visual indicator for agglutinating activity is necessary, as some *Campylobacter* sp. strains agglutinate only slowly and incompletely in immune sera, and others readily undergo autolysis or autoagglutination depending on media and cultural conditions (K. H. Wong, unpublished data). Coagglutination with *S. aureus* greatly speeds up the visibility of the reaction before autoagglutination takes place. The rapidity of the reaction and the incorporation of DNase in the antigen suspension (2, 6) made it possible to type all rough and autoagglutinating strains by coagglutination.

In the coagglutination system, the serotype cultures of *C. jejuni* and *C. coli* were determined to be of the same types as previously defined by passive hemagglutination for heat-stable antigens (8) and by slide agglutination for heat-labile antigens (6). The coagglutination procedure thus provides a simple way to identify heat-stable and heat-labile antigens without the necessity of redefining the already established serotypes, and a serotyping scheme may be constructed for *C. jejuni* and *C. coli* incorporating both heat-stable and heat-labile antigens, using one assay system.

The coagglutination test eliminates the laborious and time-consuming extraction, sensitization, and assay steps of the passive hemagglutination test. The typing sera prepared by intravenous immunization of rabbits with live serotype cultures as described by Penner and Hennessy (8) were

TABLE 2. Comparison of cross-reactions of heat-stable antigenic types by hemagglutination (HA) and coagglutination (CoA)†

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Test</th>
<th>Cross-reaction of heat-stable antigenic type strain:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P4</td>
<td>P13</td>
</tr>
<tr>
<td>P4</td>
<td>HA</td>
<td>5,120</td>
</tr>
<tr>
<td></td>
<td>CoA</td>
<td>4+</td>
</tr>
<tr>
<td>P13</td>
<td>HA</td>
<td>640</td>
</tr>
<tr>
<td></td>
<td>CoA</td>
<td>1+</td>
</tr>
<tr>
<td>P16</td>
<td>HA</td>
<td>2,560</td>
</tr>
<tr>
<td></td>
<td>CoA</td>
<td>2+</td>
</tr>
<tr>
<td>P23</td>
<td>HA</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>CoA</td>
<td>0</td>
</tr>
<tr>
<td>P36</td>
<td>HA</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>CoA</td>
<td>0</td>
</tr>
<tr>
<td>P43</td>
<td>HA</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>CoA</td>
<td>0</td>
</tr>
<tr>
<td>P50</td>
<td>HA</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>CoA</td>
<td>0</td>
</tr>
</tbody>
</table>

* HA titer values are reciprocals of the serum dilutions giving a definitive hemagglutination pattern in microtiter wells.
* Coagglutination was graded under a dissection microscope according to the approximate amount of agglutination (1+ = 25%, 2+ = 50%, 3+ = 75%, 4+ = >75%).

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**TABLE 3. Determination of heat-stable and heat-labile antigens on individual strains of *C. jejuni* and *C. coli* by coagglutination**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Heat-stable antigenic type</th>
<th>Heat-labile antigenic type</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>P4</td>
<td>4</td>
<td>17</td>
</tr>
<tr>
<td>P5+</td>
<td>5+</td>
<td>9</td>
</tr>
<tr>
<td>P6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>P9</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>P10</td>
<td>10</td>
<td>16</td>
</tr>
<tr>
<td>P16</td>
<td>16</td>
<td>17</td>
</tr>
<tr>
<td>L1</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>L2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>L4</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>L5</td>
<td>23, 36 (weak)</td>
<td>5</td>
</tr>
<tr>
<td>L6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>L17</td>
<td>16</td>
<td>17</td>
</tr>
</tbody>
</table>

* Results on heat-stable antigens by coagglutination were confirmed by passive hemagglutination.
* Heat-labile antigenic types determined by coagglutination were confirmed by slide agglutination.
adequate for the coagglutination test without absorption, as the typing results for the heat-stable antigens were confirmed by testing a heated aliquot of the cultures (Fig. 1). Extraction of bacteria is not necessary as the heat-stable antigens of whole bacteria are readily detectable by coagglutination. In their study on strain-specific and interstrain-related antigens of C. fetus, C. intestinalis, and C. jejuni, Kosunen and co-workers (4) showed that the results from line-rocket immunoelectrophoresis and rocket-line immunoelectrophoresis on cellular sonicates agreed with those obtained by coagglutination, using heated whole cells as antigens.

Preparation of monospecific typing sera for heat-labile antigens by cross-absorption (6) is difficult and time-consuming. Compared with the slide agglutination test (6), the coagglutination procedure conserves use of absorbed antisera by utilizing unabsorbed antisera to sensitize protein A-positive S. aureus for the presumptive test. Monospecific reagents are used only for final confirmation. Moreover, the volume of monospecific antibody available for typing heat-labile antigens is greatly increased by attaching the antibody in the absorbed serum to protein A-positive S. aureus cells for the coagglutination test.

The use of pooled reagents in the first step of the coagglutination system simplifies the operation by eliminating testing a culture with a large number of typing reagents and provides the flexibility of formulating the pools according to the prevalence rate of different serotypes and experimental designs. The selection and grouping of the 35 heat-stable antigenic types should detect about 83% of C. jejuni serotypes and 89.8% of C. coli serotypes (9), and the 11 heat-labile antigenic types should cover about 75% of C. jejuni/C. coli isolates in the United States. Recently, Penner and co-workers reported a simplified procedure for the passive hemagglutination test for typing heat-stable antigens, making use of serum pools (J. L. Penner, J. N. Hennessy, and R. V. Congi, Abstr. Annu. Meet. Am. Soc. Microbiol. 1984 C52, p. 245). As more antigenic types are being described and added to the many already established serotypes of C. jejuni and C. coli, pooling of type-specific antibodies in a two-step procedure would make typing of Campylobacter spp. more practical.

Once antisera of adequate quality are prepared, setting up and operating the coagglutination system are quite simple. The sensitized S. aureus cells are stable, and the antigenic activity of the Campylobacter sp. cells can be preserved in the suspension fluid for at least several months. The suspension fluid containing BSA, ions, and DNase was formulated for preserving the Campylobacter sp. cells for use as antigens in this study. The suspension fluid is not necessary for routine testing, but may be used for preserving serotype cultures as control antigens. In routine coagglutination operations, Campylobacter sp. colonies are picked from petri plates and emulsified in PBS containing DNase (1 μg/ml). After mixing thoroughly on a Vortex mixer, a portion of the culture is heated at 100°C for 1 h. Both heated and unheated portions are tested by coagglutination. Slight contamination of the test cultures with common contaminants from the air and medium (which contains 5% rabbit blood) such as bacillus and staphylococcal species is not likely to affect typing results as there is no evidence that these bacterial species contain antigens cross-reacting with those of Campylobacter spp. (K. H. Wong and S. K. Skelton, unpublished data).

These results with 68 serotype strains demonstrate that the coagglutination system for typing C. jejuni and C. coli should be practical for public health and clinical laboratories. Evaluation of the system with wild strains from disease outbreaks is under way, and further simplification of the test procedures may be possible.

LITERATURE CITED