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* spp., 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 one simple 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% CO_2. 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% $CaCl_2$, 0.01% $MgCl_2$, and 0.002% $MnCl_2$. 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 heated antigens.

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 nonspecific 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 dissection 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

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formaldehyde-treated serotype cultures (6). Both unabsorbed and absorbed sera were used in experiments on typing heat-labile antigens (see Fig. 1). Absorption of antisera for the monospecific heat-labile antigenic types used in this study was performed as described by Lior and co-workers (6).

Typing reagents for coagglutination. A 1% suspension of lyophilized cells of *Staphylococcus aureus* (protein A-positive 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 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 microscope 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 coagglutinated with cells of *S. aureus* sensitized with the *Campylobacter* 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 sensitize the *S. aureus* and on the nature of the *Campylobacter* sp. serotype antigen. *Campylobacter* 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 occurred 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 agglutination test. Although not all "rough" cultures autoagglutinated in normal sera, the autoagglutinating strains invariably showed a "rough" settlement pattern.

Typing of cultures. After the individual typing reagents and the pools were tested with the respective *Campylobacter* 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 laboratory 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-stable or heat-labile antigens as defined by Penner and Hennessy (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-reactions 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 1. Typing of *C. jujuni* and *C. coli* by coagglutination

No. of serotypes	No. of strains	No. of strains correctly typed
Heat-stable antigens[a]		
57 (used in pooling by numerical order)	57	57
35 (used in pooling by species and prevalence)	35	35
Heat-labile antigens[b]		
11 (used in pooling by prevalence)	11	11

[a] Antigenic types determined by coagglutination were confirmed by passive hemagglutination.
[b] Results by coagglutination were confirmed by slide agglutination.
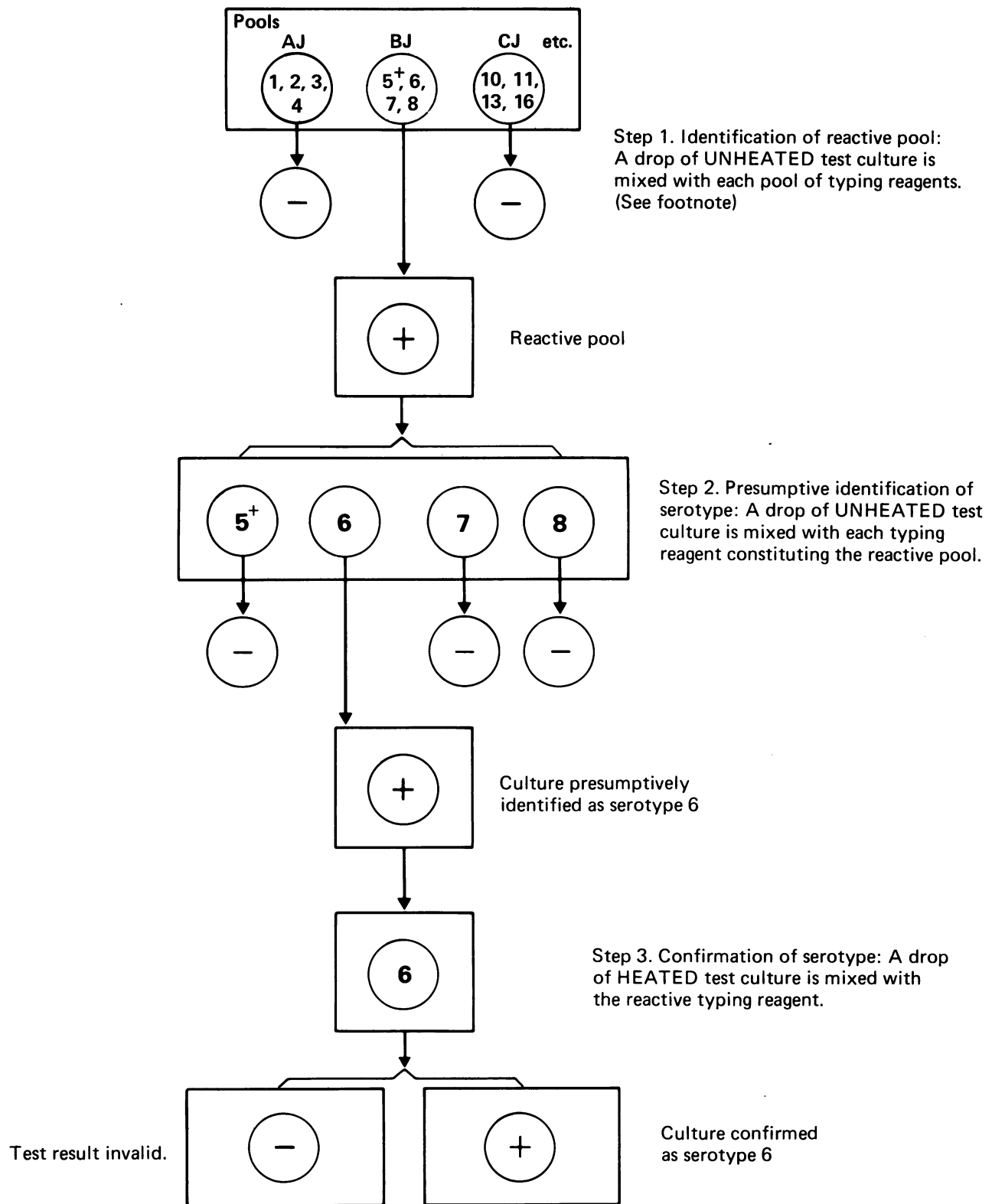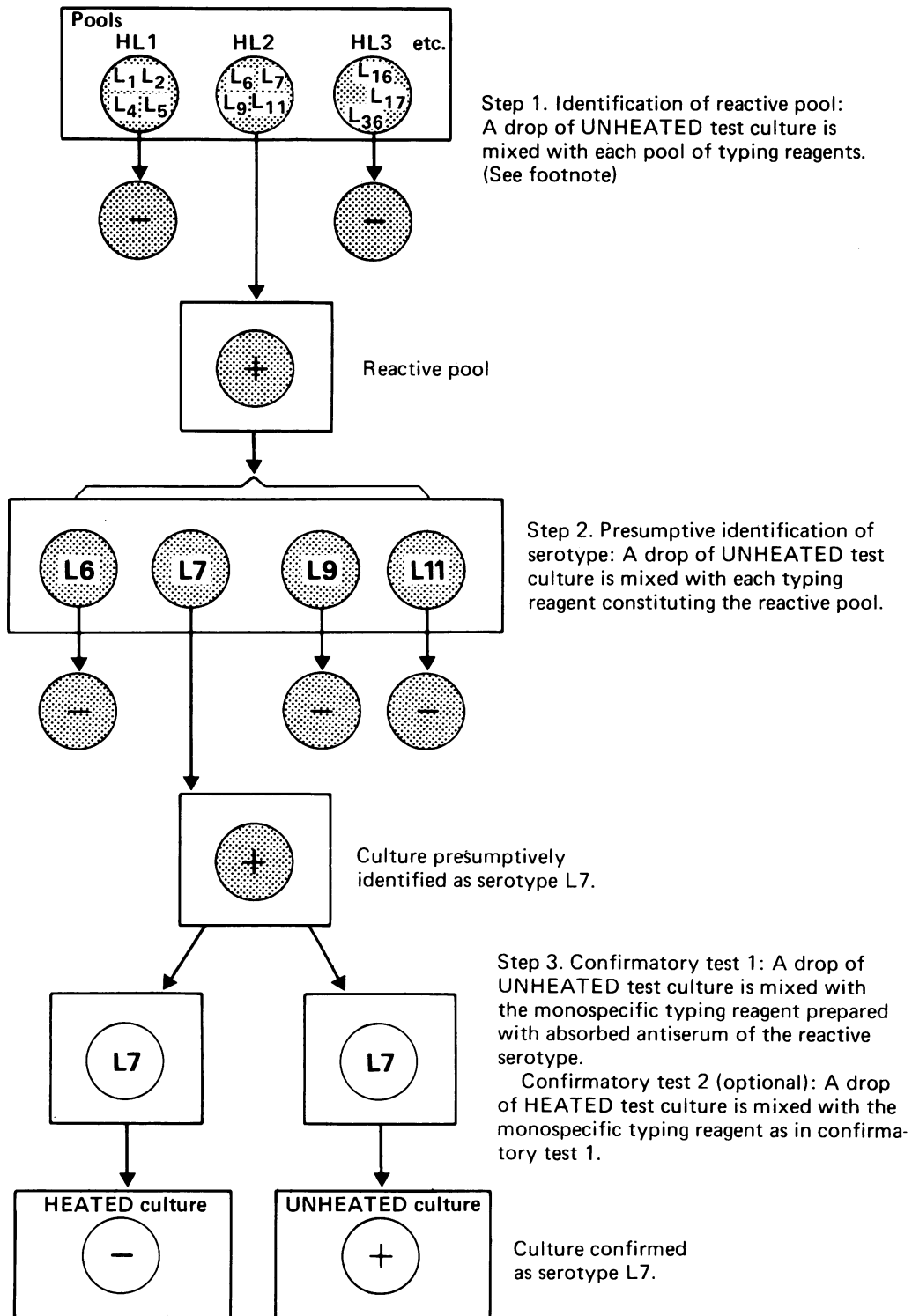
A. Typing of heat-stable antigens

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.

B. Typing of heat-labile antigens

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

Reactive pool

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 L7.

Step 3. Confirmatory test 1: A drop of UNHEATED test culture is mixed with the monospecific typing reagent prepared with absorbed antiserum of the reactive serotype.
    Confirmatory test 2 (optional): A drop of HEATED test culture is mixed with the monospecific typing reagent as in confirmatory test 1.

Culture confirmed as serotype L7.

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

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.

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