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Development of a Bacteriophage Typing System for *Campylobacter jejuni* and *Campylobacter coli*

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A bacteriophage typing system for *Campylobacter jejuni* and *Campylobacter coli* was developed with phages isolated from poultry feces. Data for phage selection were generated from a set of isolates of *C. jejuni* and *C. coli* from humans in Illinois. Selection of 14 phages from the 47 phages available was assisted by determination of the Sneath-Jaccard similarity coefficients and subsequent unweighted pair-group arithmetic averaging cluster analysis. The typing set was reproducible and stable in the 255 isolates from Illinois. Of these isolates, 94.5% were typable, with 46% represented by the four most common phage patterns. In a set of 51 isolates from humans outside of Illinois, 88.1% of the *C. jejuni* isolates were typable. Phage typing for *C. jejuni* and *C. coli* has excellent epidemiologic potential and should serve as a useful adjunct or alternative to serotyping systems in current use.

A reproducible, sensitive, and well-standardized typing system is critical to the successful investigation of outbreaks caused by *Campylobacter jejuni* and *Campylobacter coli*. Understanding of sources and means of transmission of these enteric pathogens will also improve as the discriminatory ability of *Campylobacter* typing systems increases. Identification systems of epidemiologic value based on biotyping (8, 19, 30), serotyping (20, 24), and plasmid (35) or restriction endonuclease (16) analyses have been described. Each system has methodological advantages and disadvantages. The latter two methods achieve excellent resolution of patterns suitable for typing but require facilities not available in many laboratories. Biotyping and serotyping are more available but present problems in the interpretation and resolution of typing results. Patton et al. (23) have suggested the combined use of Penner indirect hemagglutination serology (24) and Lior slide agglutination serology (20). The combined methods, used with approximately 111 different antisera, typed 98% of the strains tested.

The feasibility of an identification system based on selective lysis by bacteriophages of *C. jejuni* and *C. coli* has been examined previously by several investigators. Phages of *C. jejuni*, *C. coli*, and other *Campylobacter* species have been morphologically characterized by Bryner et al. (10–12). Although an individual *C. jejuni* phage has been used to characterize isolates from children with gastroenteritis (7) and small sets of *C. jejuni* and *C. coli* phages have been used to test for lytic activity in isolates from humans and animals (11, 27), a standardized bacteriophage typing system of practical value in epidemiologic studies of human *C. jejuni* and *C. coli* infections has not been developed.

Our goals were to isolate a wide variety of phages from natural sources, to select an optimal typing set by employing current numerical taxonomic methods, and to establish a simple typing system which would retain as much epidemiologic discrimination as possible. In this report, we describe the use of a set of local (Illinois) *C. jejuni* and *C. coli* isolates to establish the typing set and contrast its performance with a group of cultures of international origin.

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MATERIALS AND METHODS

General methods. Unless otherwise noted, all *Campylobacter* cultures, phage propagations, and phage typing experiments were incubated at 42°C for 24 h in a microaerophilic atmosphere consisting of 5% O_2, 10% CO_2, and 85% N_2. All centrifugation was done at 2,000 × *g* for 25 min at 4°C, and all filtrations were through a 0.45-μm (pore size) filter (Millipore Corp., Bedford, Mass.).

Media. Brucella broth (BB; Oxoid Ltd., London, England) was the base for all of the media used, and all media were sterilized for 15 min at 121°C. Pure cultures of *Campylobacter* isolates were grown on BB-sheep blood agar plates. Campy-BAP plates (6) were used when a selective medium was required. Medium for culture storage at −65°C contained BB and 15% glycerol.

All media for bacteriophage typing and storage were made in our laboratory from BB and modified by the addition of 0.01 M $MgSO_4$ and 0.001 M $CaCl_2$. Modified base agar plates contained 1.5% agar (Difco Laboratories, Detroit, Mich.), and 3-ml soft overlays contained 0.7% agar. Modified BB (MBB) was used for bacterial suspension, phage suspension, and phage storage. Large quantities of agar and BB were purchased and tested before routine use to rule out potential problems in typing reproducibility, as Pruneda and Farmer (26) have described.

***Campylobacter* isolates.** All isolates used were of human origin. A total of 255 unrelated isolates from Illinois (*C. jejuni*, 240 isolates; *C. coli*, 10 isolates; 5 hippurate-intermediate isolates), isolated from 1981 to 1984, were obtained from the Enteric Section of the Illinois Department of Public Health (185 isolates), the Chicago Health Department (42 isolates), Evanston Hospital, Evanston, Ill. (14 isolates), University of Illinois Hospitals, Chicago, Ill. (9 isolates), and West Suburban Hospital, Oak Park, Ill. (5 isolates).

A total of 51 isolates from outside of Illinois (*C. jejuni*, 42 isolates; *C. coli*, 9 isolates) were provided by the following contributors: H. Lior, Laboratory Centre for Disease Con-

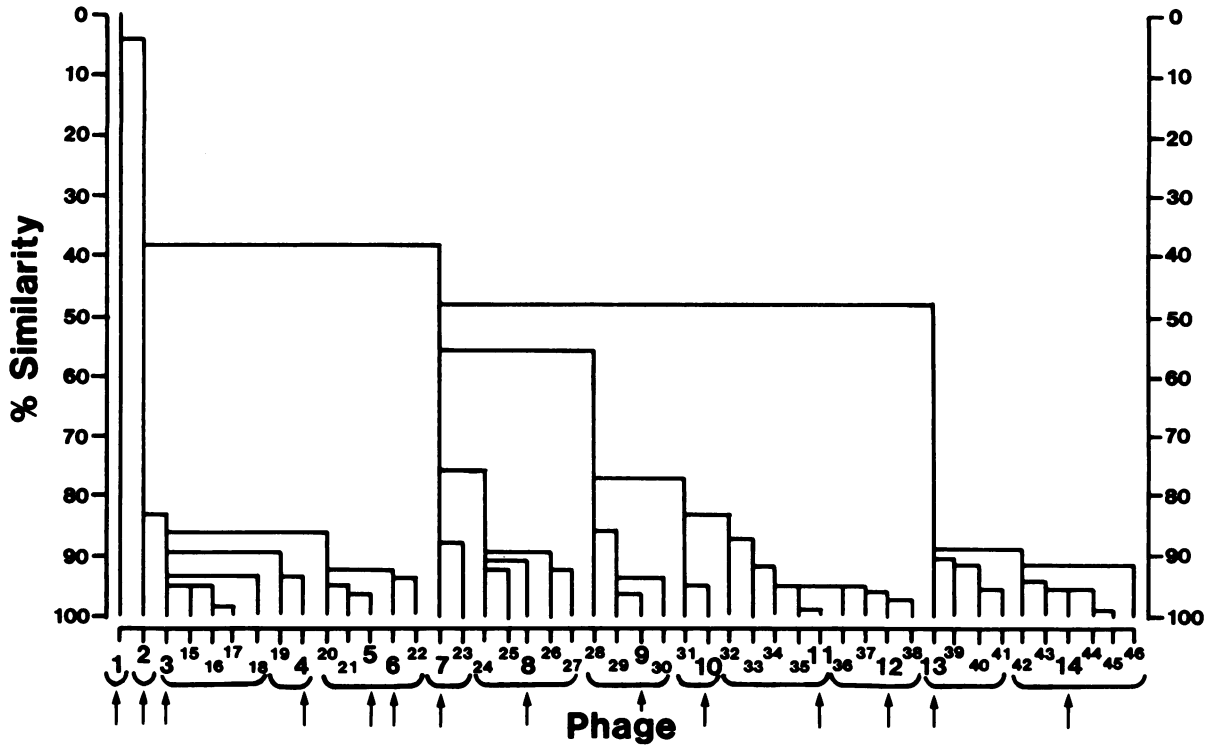

FIG. 1. Selection of the 14-phage typing set. The phenogram (tree diagram) depicts relationships among the 46 phages as determined by UWPGMA. Vertical axes indicate the maximum similarity between phage pairs or among clusters, as indicated by the horizontal lines connecting them. The 13 clusters of similar phage groups are marked with braces, and arrows point to phages 1 through 14, the phages chosen as members of the final typing set. See text for details.

trol, Ottawa, Ontario, Canada (18 isolates); T. W. Steele, Institute of Medical and Veterinary Service, Adelaide, South Australia (10 isolates); W. L. Wang, Veterans Administration Hospital, Denver, Colo. (8 isolates); J. Bryner, National Animal Disease Center, U.S. Department of Agriculture, Ames, Iowa (5 isolates); E. Falsen, Institute of Medical Microbiology, Gothenburg, Sweden (4 isolates); V. D. Bokkenheuser, St. Luke's Hospital Center, New York, N.Y. (3 isolates); and J. Dekeyser, National Institute of Veterinary Research, Brussels, Belgium (3 isolates).

Although each of these isolates had been confirmed as *C. jejuni* or *C. coli* by the original investigators, a Gram stain, dark-field characteristic motility check, and hippurate hydrolysis test by the method of Hwang and Ederer (14) were performed on a 24- to 36-h growth from each isolate immediately before use in phage typing experiments, and all were reconfirmed by us.

Bacteriophages. One *C. jejuni* (c/958, ovine origin) and one *C. coli* (Cc/1491, porcine origin) bacteriophage were provided by J. Bryner. These two phages have been previously described (28).

Isolation of bacteriophages from poultry manure. Samples of fresh poultry manure were collected in southern Wisconsin, northern Illinois, and Missouri. Suspensions of each sample in MBB (1:10) were allowed to settle at 4°C for 45 min. The suspensions were filtered through coarse filter paper and centrifuged. Supernatants were filtered and loaded into duplicate 1-ml syringes with 22-gauge, 1.5-in (3.81-cm) needles.

Indicator lawns for a test panel of 27 representative cultures were prepared by making bacterial suspensions in MBB from 36-h growth on BB-blood agar plates. Suspension turbidity was equal to a McFarland no. 1 nephelometer standard (22). Each 3-ml soft agar overlay was inoculated at 50 to 55°C with 0.5 ml of bacterial suspension, mixed, and poured over the surface of a modified base agar plate. The multisyringe phage applicator described by Farmer (2) was used to spot the indicator lawns with duplicate 10-μl drops of each filtrate. Plates were dried with the covers off for 5 min under a laminar flow hood and incubated.

A total of 45 phages suitable for typing were isolated from four different sources of poultry manure. Attempts to isolate phages from cow or rabbit feces, local sewage sludge, or primary effluent were unsuccessful, as were attempts to induce lysogenic *Campylobacter* isolates in broth culture with mitomycin C.

Purification of phages and stock production. If plaques were present at the filtrate application site for a specific filtrate-indicator culture combination, 0.5 ml of filtrate was mixed with 10 ml of a 24-h MBB static culture of the appropriate indicator strain (initial inoculum, 3×10^6 bacteria). The filtrate and bacterial suspension were reincubated to allow phage propagation for 24 h, centrifuged, and filtered. Soft-agar overlays of the indicator host culture were made as described above, but 0.5 ml of the propagated filtrate was added directly to the overlay, and plates were not dried. After incubation, individual plaques were selected with sterile Pasteur pipettes into 10 ml of 24-h MBB static cultures of the appropriate indicator culture, reincubated for 24 h, centrifuged, and filtered. For each phage, 3 cycles of plaque purification were performed in its indicator host to ensure homogeneity of phage stocks. Phage stocks were prepared by the soft-agar overlay method of Swanstrom and Adams (34). Stocks were titrated and stored at −65°C.

TABLE 1. Phage typing patterns found in 255 *C. jejuni* and *C. coli* isolates from Illinois

Phage pattern	No. of isolates[a]	% of total
67000	52	20.4
67773	25	9.8
06000	20	7.8
67770	20	7.8
00000[b]	14	5.5
67470	8	3.1
67570	7	2.7
47000	6	2.3
66000	4	1.6
00463	3	1.2
04000	3	1.2
10373	3	1.2
47773	3	1.2
67003	3	1.2
67070	3	1.2
67303	3	1.2
67463	3	1.2
67473	3	1.2

[a] Two isolates each (total, 9.4%: 00460, 00470, 00770, 27000, 46000, 47003, 63000, 67300, 67370, 67372, 67460, 67772. One isolate each (total, 18.8%): 00030, 00110, 00203, 00303, 00373, 00570, 00773, 02000, 07000, 07770, 10773, 20573, 20773, 25173, 27173, 27470, 27471, 27773, 40770, 42000, 43003, 47002, 47300, 47340, 47352, 62471, 63463, 66470, 66572, 66370, 66770, 67001, 67010, 67040, 67050, 67060, 67110, 67170, 67172, 67363, 67373, 67471, 67573, 67670, 67673, 67771, 72573, 77173.

[b] Untypable.

Bacteriophage typing. The routine test dilution for *C. jejuni* and *C. coli* phage typing was defined as 10^6 PFU/ml, according to the recommendations of Baker and Farmer (2). When the multisyringe phage applicator was used with 22-gauge needles, each test applied 10^4 PFU as a 10-μl drop to the indicator lawn. Phages at routine test dilution were applied in duplicate with the multisyringe phage applicator to indicator lawns made as described for initial phage isolation. After drying for 5 min, plates were incubated and read after 18 h.

The presence of 50 or more plaques was recorded as a positive result; 49 or fewer plaques were read as a negative result. The choice of 50 PFU as a minimum for a positive reaction was based on descriptive analysis of the frequency distribution of all results with less than confluent lysis. The frequency distribution resembled a U-shaped curve; most countable reactions approached 0 or 100 PFU, and the lowest frequencies of results were those of intermediate values (50 PFU). If turbidity developed in the center of confluent lysis, the result was still considered positive. Typing data were reduced to binary form with no intermediate scores; if either one of the duplicate tests was positive, the result was considered positive.

An octal code similar to that used to record scores in the API 20E system (Analytab Products, Plainview, N.Y.) reduced the 14 reactions of the final phage typing set to a five-digit type pattern. A type pattern for any given isolate contained its 14 binary (positive or negative) phage reactions arranged in ascending numerical order. The 14 reactions were then divided into four groups of 3 reactions (phages 1 through 3, 4 through 6, 7 through 9, and 10 through 12) followed by one group of 2 reactions (phages 13 and 14). If positive, the first, second, and third reactions in a group were assigned values of 1, 2, and 4, respectively. Negative reactions in any position were assigned 0 values. Values were added for each group of three phages (and for phages 13 and 14); these five sums, ranging from 0 to 7, became the five digits of the octal code for the type pattern of a specific isolate.

Selection of the typing set. Numerical taxonomic techniques (33) were used to assist in the selection of phages for a final typing set. Our objective was to select phages with the widest diversity of reactions and with maximal ability, as a group, to type *C. jejuni* and *C. coli* cultures. The Numerical Taxonomy System of Multivariate Statistical Programs (NTSYS) (29), a large software package, was adapted to run on an IBM 3081D computer. To generate data for computer-assisted selection of the typing set, all 45 phages isolated and the two phages from Bryner were tested against a systematic random sample of 150 of a total of 255 *C. jejuni* and *C. coli* isolates from Illinois. The binary raw data matrix, 150 *C. jejuni* and *C. coli* isolates by 47 phages, was reduced to a symmetric matrix (47 by 47) with the Jaccard-Sneath similarity index ($S_{J\text{-}S}$) (32). The matrix of $S_{J\text{-}S}$ values was used for three subsequent cluster analyses: single linkage clustering, complete linkage clustering, and a "compromise" method, unweighted pair-group arithmetic average clustering (UWPGMA). Cophenetic correlation coefficients measured the degree of agreement between the original $S_{J\text{-}S}$ matrix and the matrix of maximal similarities (cophenetic values) derived from each cluster technique; optimality of the clustering techniques was evaluated in this manner. Phenograms (tree diagrams of phage clusters based on phenetic relationships) were constructed to visualize groups of similar phages. Final selection of the 14 phages was based on clusters formed in these analyses. Within a cluster of similar phages, selection of the phage for use in the typing set was made with preference given to easily propagable phages.

Reproducibility of typing method. To determine reproducibility, a 33% systematic random subsample of the 150 isolates from Illinois was retyped. Reproducibility was calculated as the proportion of these 50 isolates with less than a two-phage difference between the first and second typing patterns.

Consistency of typing pattern frequencies in isolates from Illinois. The 105 isolates from Illinois not chosen in the original systematic random sample of 150 were typed with the 14-phage typing set. The frequencies of phage patterns in this set of isolates and in the original set of 150 isolates were compared by using a χ^2 test to detect any significant difference between typing pattern frequencies in these two similar sets of isolates.

Phage typing pattern frequencies in an international set of isolates. The 51 isolates of international origin described above were phage typed, and the frequencies of the typing patterns were determined. Because these isolates represented a collection from several contributors and the proportions of the two *Campylobacter* species were dissimilar to those found in community surveys, we compared the proportions of untypable isolates in the Illinois and non-Illinois groups, controlling for the effect of *Campylobacter* species.

TABLE 2. Reproducibility of typing patterns in 50 *C. jejuni* and *C. coli* isolates

No. of pattern differences[a]	No. of isolates
0	36
1	11
2	2
4	1

[a] Differences between two typings of the same isolate.

TABLE 3. Comparability of phage typing patterns in two groups of *C. jejuni* and *C. coli* isolates from Illinois[a]

Pattern	No. of isolates (% of total) in:	
	Group 1	Group 2
67000	33 (22.0)	19 (18.1)
67773	16 (10.7)	9 (8.6)
06000	10 (6.7)	10 (9.5)
00000[b]	9 (6.0)	5 (4.8)
67770	8 (5.3)	12 (11.4)
All others[c]	74 (49.3)	50 (47.6)
Total	150	105

[a] $\chi^2 = 4.52$ ($P = 0.48$).
[b] Untypable.
[c] Frequencies too low to distinguish statistically.

The Breslow-Day test for homogeneity was performed to validate a Mantel-Haenszel χ^2 test and odds ratio (9).

RESULTS

Selection of the typing set. The three phage cluster analyses generated by NT-SYS gave similar results, which were also consistent with phage groups suggested by principal components analysis. The cophenetic correlation values were 0.97 for UWPGMA, 0.96 for complete linkage analysis, and 0.94 for single linkage analysis. These values indicate a high degree of agreement with $S_{J\text{-}S}$ values and are a measurement of methodological optimality.

The tree diagram (phenogram) from UWPGMA is shown in Fig. 1. Vertical axes indicate cophenetic values, the percent similarity between two phages or clusters as determined by the cluster analysis; the horizontal line connecting two phages or clusters shows their maximal relatedness level as expressed by percent similarity. After the UWPGMA phenogram was compared with phenograms from single and complete linkage clustering, the 46 phages were found to be segregated into the 13 marked clusters of similar phage groups. The threshold at which clusters were considered to be separate from the rest of the tree (phenon line) was approximately 85 to 90% similarity; that is, groups of phages that were still connected below this 85 to 90% horizontal band were generally considered distinct clusters. With one exception, one representative phage was chosen from each cluster to form the final typing set; phage 6 was retained in the final set because of its ability to type increased numbers of both *C. jejuni* and *C. coli*.

The members of the final typing set were arbitrarily named phages 1 through 14. Whereas the *C. jejuni* phage from Bryner became phage 1 of the typing set, his *C. coli* phage was inactive on all isolates tested and was not used in subsequent analyses. Eight *C. jejuni* strains were used as hosts for phages 1 through 14: host 177 (Bryner 958) for phage 1; host 328 for phages 2 and 5; host 22 for phages 3 and 6; host 101 for phage 4; host 106 for phages 7, 12, and 14; host 305 for phage 8; host 263 for phages 9 and 13; and host 86 for phages 10 and 11.

Phage patterns found in Illinois. A total of 77 phage patterns were identified among the 255 unrelated Illinois *C. jejuni* and *C. coli* isolates. Their numerical and percentage distributions are shown in Table 1. Untypable isolates are represented by 00000. Nearly half (45.9%) of the isolates fell into the four most common patterns. A total of 60 patterns may be considered rare (in this place and at this time), since they each identified less than 1% of the isolates tested. Altogether, 94.5% of the *C. jejuni* and *C. coli* isolates were typed.

Reproducibility of typing method. Table 2 lists the number of pattern differences found in the 50 isolates which were typed twice. If reproducibility is defined as the percentage of isolates whose typing pattern differed by one phage or less when typing was repeated, then the typing method was 94% reproducible.

Consistency of typing pattern frequencies in isolates from Illinois. In the two groups of typed *C. jejuni* and *C. coli* isolates from Illinois (the original sample of 150 and the group of 105 not initially chosen), the frequencies of the identified typing patterns were similar (Table 3). Phage typing pattern frequencies appeared to be the same in different groups of isolates collected at about the same time from the same geographic region.

Pattern frequency distribution of *C. jejuni*, *C. coli*, and hippurate-intermediate isolates from Illinois. Table 4 compares the frequencies of phage patterns among *C. jejuni* and *C. coli* isolates. The 14 phages successfully typed 95.8% of the *C. jejuni* isolates and 60% of the 10 *C. coli* isolates. The five hippurate-intermediate isolates were all typable.

TABLE 4. Most common type frequencies in 255 unrelated *C. jejuni* and *C. coli* isolates from Illinois

Type pattern	No. of isolates (% of total) of:		
	C. jejuni ($n = 240$)	*C. coli* ($n = 10$)	Total both species[a] ($n = 255$)
67000	48 (20.0)	2 (20.0)	52 (20.4)
67773	25 (10.4)		25 (9.8)
06000	20 (8.3)		20 (7.8)
67770	19 (7.9)	1 (10.0)	20 (7.8)
00000[b]	10 (4.2)	4 (40.0)	14 (5.5)
67470	8 (3.3)		8 (3.1)
67570	6 (2.5)		7 (2.7)
47000	6 (2.5)		6 (2.4)
66000	3 (1.3)		4 (1.6)
All others[c]	95 (39.6)	3 (30.0)	99 (38.8)

[a] Five cultures with intermediate hippurate reactions were deleted from species totals but included in this total. The patterns found were 67000 (two isolates), 00470, 66570, and 66000 (one isolate each).
[b] Untypable.
[c] Numbers of distinct type patterns were 67, 3, and 69 for *C. jejuni*, *C. coli*, and both species, respectively.

TABLE 5. Most common type pattern frequencies in 51 unrelated *C. jejuni* and *C. coli* isolates from outside of Illinois[a]

Type pattern	No. of isolates (% of total) of:	
	C. jejuni ($n = 42$)	*C. coli* ($n = 9$)
67000	8 (19.0)	3 (33.3)
00000[b]	5 (11.9)	5 (55.6)
67773	5 (11.9)	
67463	4 (9.5)	
67772	2 (4.8)	
67470	2 (4.8)	
24000	2 (4.8)	
All others[c]	14 (33.3)	1 (11.1)

[a] See text for list of cultures.
[b] Untypable.
[c] Numbers of distinct type patterns were 14 and 1 for *C. jejuni* and *C. coli*, respectively.

TABLE 6. Proportions of untypable isolates among those from Illinois and those from outside of Illinois, controlling for *Campylobacter* species[a]

Isolate origin	No. (%) of isolates of:			
	C. jejuni		*C. coli*	
	Untypable	Typable	Untypable	Typable
Illinois	10 (4.2)	230 (95.8)	4 (40.0)	6 (60.0)
Non-Illinois	5 (11.9)	37 (88.1)	5 (55.6)	4 (44.4)

[a] Mantel-Haenszel $\chi^2 = 4.019$ ($P = 0.045$). Mantel-Haenszel estimate of the common odds ratio; 2.03 (95% confidence interval, 1.02 to 4.08). Breslow-Day test for homogeneity; $\chi^2 = 0.22$ ($P = 0.64$).

Phage typing pattern frequencies in an international set of isolates. The distribution of major type patterns among an international set of isolates was similar but not identical to that found for the isolates from Illinois (Table 5). Six type patterns accounted for roughly half of the isolates tested. As was the case with the isolates from Illinois, fewer *C. coli* isolates (44.4%) than *C. jejuni* isolates (88.1%) were typable.

The proportions of untypable isolates among those from Illinois and those from outside of Illinois were compared (Table 6). When the effect of differing *Campylobacter* species was removed, it was shown statistically that isolates from places other than Illinois (part of the region from which the typing phages were isolated) were more likely to be untypable; the odds that an isolate was untypable were doubled if the isolate was not from Illinois.

DISCUSSION

The bacteriophage typing system described is a reasonably simple and reproducible means of typing *Campylobacter* isolates from Illinois. Its use for typing *Campylobacter* isolates from other areas is also promising. The diversity and number of patterns found suggests excellent epidemiologic potential for the typing set, since evidence for epidemiologic relatedness is much more convincing if a group of potentially related isolates share a rarely found phage typing pattern.

For *C. jejuni*, phage typing is comparable in simplicity and typability to current serotyping systems. We have made progress in phage typing *C. coli*, which represented 3.9% of our routine isolates from humans in Illinois and 2 to 3.2% of the isolates in other surveys (15, 17). Since none of the phages isolated typed *C. coli* exclusively or predominantly, this typing system appears to differ from the serotyping system of Penner et al. (25), in which thermostable antigens are comparatively species specific. Further development of the set should include determination of *C. coli*-specific types, perhaps by addition of a *C. coli* species-specific phage produced by mutagenesis or isolation from other natural sources such as swine (13, 27). The data presented also suggest that there is some local specificity to the host ranges of the phages and that the typability of isolates from other parts of the world could be improved by the addition of phages from a wider geographic region to the typing set.

We and other investigators (2, 8) have evaluated or considered some variables affecting typing reproducibility. In addition to variations in typing results caused by time or methodology, *Campylobacter* serotyping and phage typing systems have the potential for modification based on unanticipated antigenic changes. Phage conversion, plasmids, and lysogeny, for example, have caused well-documented instances of changes in serotypes or phage types for *Campylobacter* species (21) and other organisms (5, 31). The impact of these phenomena on the performance and reproducibility of *Campylobacter* typing systems needs to be determined. The 94% reproducibility calculated for the typing set described in this report allows for a one-phage difference in repeat typings, which is considered reasonable for *Staphylococcus aureus* and some other typing schemes (1). If absolute duplication of initial typing results is considered necessary, the reproducibility of the typing system drops to 72%. Additional reproducibility testing with generalized kappa-type statistics (18), for example, might help to modify, eliminate, or substitute for individual phages, antisera, or biochemical reactions which exhibit excessive variability in a typing system.

Our approach to the selection of this typing set was based on the methodological analyses of Bergan (3, 4), who determined the most effective algorithms of numerical taxonomy for the selection of *Pseudomonas aeruginosa* phages. The NT-SYS software package by Rohlf et al. (29) eliminates most of the effort required to computerize these techniques and is straightforward to use. In the case of this typing set, cophenetic correlation coefficients suggest that the clustering methods used were similar in efficacy, but when phages from different locations are combined, the need to select a best clustering method may be more critical. A table of similarity coefficients and a phenogram depicting the relatedness of phages will prove extremely useful in determining whether differing patterns seen in an outbreak could indeed be quite closely related. Finally, although NT-SYS can handle some scoring systems other than binary ones, the reduction of these typing data to simple binary form still resulted in a fully operational, reproducible typing system. We recommend that other investigators interested in forming or expanding bacteriophage typing sets increase the resolution and efficiency of their selection by adopting current numerical taxonomic techniques similar to those implemented in NT-SYS.

The phage typing system described may differ from serotyping systems in its response to *Campylobacter* receptors and antigenic determinants; isolates belonging to a commonly found serogroup may, therefore, have a rarely found phage typing pattern and vice versa. Bacteriophage typing for *C. jejuni* and *C. coli* offers a useful complementary addition to the serotyping systems currently in epidemiologic use.

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