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New Bacteriophage Typing System for *Yersinia enterocolitica*, *Yersinia kristensenii*, *Yersinia frederiksenii*, and *Yersinia intermedia*: Correlation with Serotyping, Biotyping, and Antibiotic Susceptibility

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Yersinia enterocolitica is listed as a single species in *Bergey's Manual of Determinative Bacteriology*, but has recently been split into "true" *Y. enterocolitica*, *Y. kristensenii*, *Y. intermedia*, and *Y. frederiksenii*. From 48 bacteriophages isolated from raw sewage, 24 were chosen as being the most useful for differentiating strains within the four *Yersinia* species. The composite set of 24 phages typed 92% of 236 *Y. enterocolitica* strains, 100% of 16 *Y. kristensenii* strains, 97% of 29 *Y. frederiksenii* strains, and 90% of 20 *Y. intermedia* strains. The most common phage type in any of the groups contained 22% of the strains tested, but most of the phage types contained <5% of the strains. The new typing schema was tested in three outbreaks of *Y. enterocolitica*, and the results agreed well with serotyping and epidemiological findings. In the same outbreaks, biotyping (API 20E profiles; Analytab Products, Plainview, N.Y.) and antibiograms were less reliable markers and probably should be used only in conjunction with serotyping or phage typing or both. Caution should be used in identifying cultures of *Y. frederiksenii* and *Y. intermedia* with the API 20E system, since the tests at 37°C for L-rhamnose and melibiose fermentation are often delayed past 24 h, which is the cut-off point for the final reading in the API system. There were distinct differences in the susceptibilities of *Y. enterocolitica* and *Y. kristensenii* to ampicillin, carbenicillin, and cephalothin, which adds further support for classifying the latter as a separate species.

Before 1975, *Yersinia enterocolitica* was regarded as a single species that could be separated into biochemical groups (biogroups, biovars, or biotypes). DNA-DNA reassociation has shown that strains formerly identified as *Y. enterocolitica* can be split into four distinct hybridization groups (7, 8, 11–13, 16). These four groups correspond to "true" (in the narrow sense) *Y. enterocolitica*, *Y. kristensenii*, *Y. intermedia*, and *Y. frederiksenii* and can be differentiated on the basis of acid production from sucrose, L-rhamnose, raffinose, and melibiose (see Table 1). *Y. enterocolitica* often causes human illnesses such as gastroenteritis, septicemia, arthritis, mesenteric lymphadenitis, and terminal ileitis and has been involved in several outbreaks (2, 9, 20, 28, 30, 31). *Y. frederiksenii* and *Y. intermedia* apparently are not intestinal pathogens, but have been associated with wound and skin infections (10, 12, 22). *Y. kristensenii* is most often isolated from the environment and has seldom been implicated in human disease (8). Because the recent nomenclatural changes correlate with pathogenicity, it is important to distinguish *Y. enterocolitica* from the other three species.

Laboratory methods to differentiate strains of *Y. enterocolitica* have been limited to biochemical tests (biotyping), the determination of O antigens (serotyping), antibiotic susceptibility (the antibiogram), and bacteriophage susceptibility (9, 30). In the United States, most isolates involved in human infections have been serogroup O 8, but this O group is rare in Canada and Europe, where O 3 and O 9 are the most common. However, sensitive methods for differentiating strains within these common O groups have not been available. The recognition of *Y. kristensenii*, *Y. frederiksenii*, and *Y. intermedia* as species is so recent that serological methods have not been developed. Differences in susceptibility to certain antibiotics have been

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used by several investigators to assess the relationship of different *Y. enterocolitica* strains to each other and to *Y. pseudotuberculosis* (17, 21, 23). Two bacteriophage typing systems developed in Europe have been used to relate certain biotypes and serogroups of *Y. enterocolitica* to particular diseases or animal hosts, but these bacteriophages do not usually lyse any of the serogroup O 8 isolates that are common in the United States (24–27).

Y. enterocolitica strains have been well studied, but much less is known about the three new species. Because of the recent nomenclatural changes and growing awareness of the association between *Y. enterocolitica* and human illness, additional laboratory methods are needed. The purposes of this study were to develop a practical bacteriophage typing system for *Y. enterocolitica, Y. kristensenii, Y. intermedia,* and *Y. frederiksenii* and to compare phage typing with serotyping, biotyping, and antimicrobial susceptibility.

MATERIALS AND METHODS

General. Unless exceptions are given, the following statements hold throughout this paper: all experiments were done in the Enteric Section, Centers for Disease Control, Atlanta, Ga.; the temperature of incubation was 36 ± 1°C; water refers to glass-distilled water; commercial media were used whenever possible ("from individual ingredients" or "was made with" appear if a commercial medium was not used); media were sterilized in an autoclave at 121°C for 15 min; optical density was measured in a Bausch and Lomb Spectronic 20 spectrophotometer at 650 nm in 13- by 100-mm disposable glass tubes; filter sterilization was through a 0.22-μm nitrocellulose filter; refrigeration was at a temperature of 5 ± 1°C; an "overnight culture" refers to a 17- to 24-h-old stationary-phase culture with 10^8 to 10^9 organisms per ml; and "antibiotic" refers to true antibiotics and to synthetic antimicrobial agents.

Media and reagents. Trypticase soy agar (TSA) and Trypticase soy broth (TSB) and prepared Mueller-Hinton agar plates (150 by 15 mm) were from BBL Microbiology Systems (Cockeysville, Md.) and were prepared according to instructions on the bottle. TSB/5 was made with 6.0 g of TSB and 1,000 ml of distilled water. Blood agar contained 30 g of TSA, 50 ml of sheep blood, and 950 ml of distilled water. Ion agar contained 4 g of Ionagar no. 2 (Oxoid Limited, London, England) and 1,000 ml of distilled water. Skim milk for freezing cultures contained 100 g of skim milk (Oxoid) and 1,000 ml of distilled water (autoclaved at 121°C, 10 min). Triple sugar iron agar, motility test medium, and broth for esculin hydrolysis were made according to directions outlined by Edwards and Ewing (18), except that the triple sugar iron agar had an additional 5 g of agar added per liter.

The API 20E system (Analytab Products, Plainview, N.Y.) was used for preliminary screening of all isolates. Eight additional carbohydrate fermentation media were made according to Edwards and Ewing (18), except that Andrade's indicator contained 0.2 g instead of 0.5 g of acid fuchsin per 100 ml of distilled water. The concentrations of carbohydrates used were as follows: sucrose, 1.0%; L-rhamnose, 0.5%; raffinose, 0.5%; α-methyl-D-glucoside, 0.5%; melibiose, 0.5%; salicin, 0.5%; lactose, 1.0%; and D-xylose, 1.0%.

Bacterial strains. Three hundred six isolates were used in this study. Two hundred eighty were obtained from two collections at the Centers for Disease Control: 182 from the Enteric Section, Bureau of Laboratories; and 98 from James C. Feeley, Special Pathogens Laboratory Section, Bureau of Epidemiology. Seventeen isolates were from E. J. Bottone, Mount Sinai Hospital, New York, N.Y., and nine isolates were from H. Bercovier, Institute Pasteur, Paris, France. All isolates had been identified in the laboratories from which they were received as "*Yersinia*," *Y. enterocolitica*, "atypical *Y. enterocolitica*-like," or with a similar designation. They were subcultured to fresh TSA slants or were streaked to TSA plates for single-colony isolation. Working cultures were prepared by inoculating fresh TSA slants in 13- by 100-mm screw-capped tubes, incubating overnight to ensure viability, sealing with sterile butyl rubber (White, no. 000) stoppers, and storing at room temperature (17 to 27°C) in the dark. All subsequent tests were done with these "working" cultures. Frozen stock cultures were prepared from 18- to 24-h TSA slant cultures as follows: about 1 ml of skim milk was added to growth on a 24-h TSA culture, and bacterial growth from the agar slant was gently dislodged with the pipette tip. The suspension was transferred to a sterile 4-ml polypropylene screw-capped Cryotube (Vangard International, Inc., Neptune, N.J.) and slowly frozen by placing the vial directly in a −70°C freezer (Revco, Inc., West Columbia, S.C.).

Biochemical characterization. A standardized inoculum was used for API 20E strips and conventional carbohydrate media. Growth was transferred with a cotton swab from overnight TSA slant cultures to 5 ml of sterile distilled water (pH 7.0) in 13- by 100-mm screw-capped tubes. Bacteria were added until the suspension equaled an optical density of 0.1. This suspension was visually equivalent to a 0.5 Mcfarland barium sulfate standard used for antibiotic susceptibility testing and was equal to about 10^8 bacteria per ml as determined by plate count. This was called the standard turbidity. New instructions (March 1978) sent with the API strips recommended 0.85% saline as the suspending medium, but since this recommendation came after the study was well advanced, we continued to use sterile distilled water. The API strips were incubated overnight, reagents were added, and the results were interpreted according to the manufacturer's directions.

The conventional carbohydrate fermentation media and esculin broth were inoculated with about 0.1 ml of the distilled water suspension that had been prepared for the API 20E strip. The tubes were incubated, and reactions were read at 1, 2, 3, 5, and 7 days. Lactose, D-xylose, and motility media were inoculated in duplicate and incubated at both 36 and 25°C. Any change in the indicator from colorless to pink or red (pH < 6.2) was considered a positive fermentation.

The 306 isolates were tentatively identified by their API 20E biochemical profiles and then placed into one of the four *Yersinia* groups on the basis of their fermentation reactions, for sucrose, L-rhamnose, raffi-

TABLE 1. *Biochemical reactions (36°C) used to differentiate the four Yersinia species (6, 10)*

Species	Acid production from:			
	Sucrose	L-Rhamnose	Raffinose	Melibiose
Y. enterocolitica	+[a]	–	–	–
Y. kristensenii	–	–	–	–
Y. frederiksenii	+	+	–	–
Y. intermedia	+	+	+	+

[a]Symbols: + = positive within 7 days, – = negative at 7 days.

nose, and melibiose in conventional tube tests (18). The definitions of the four species are given in Table 1. The reactions listed are based on incubation at 36°C for 7 days. The reactions for *Y. intermedia* and *Y. frederiksenii* can also be done at 25°C and will usually become positive within 24 h. This lower temperature can be used to provide an earlier identification when a culture is thought to be *Y. enterocolitica*, *Y. frederiksenii*, or *Y. intermedia*. On the basis of these definitions the *Yersinia* strains were identified as follows: 241 *Y. enterocolitica*, 16 *Y. kristensenii*, 29 *Y. frederiksenii*, and 20 *Y. intermedia*.

Antibiotics. The antibiotic susceptibility pattern (antibiogram) of each isolate was determined on Mueller-Hinton agar (36°C) by the standardized single-disk method of Bauer et al. (4, 5). *Staphylococcus aureus* derived from ATCC 25923 and *Escherichia coli* derived from ATCC 25922 were included as quality control strains. Plates were incubated overnight, and the zones of complete inhibition were measured to the nearest millimeter.

Serological typing. Serological typing by agglutination in 96-well (0.4 ml each) plastic dishes had previously been done by James C. Feeley, Bureau of Epidemiology, Centers for Disease Control. Formalinized cells (0.6% Formalin) were washed twice, centrifuged at 733 × *g*, and adjusted to an optical density of 0.2 at 420 nm (Bausch and Lomb spectrophotometer, model 20; light path = 11 mm) in 0.85% NaCl with 0.01 M sodium phosphate (pH 7.6). This suspension was added to equal volumes (0.025 ml) of serially diluted (twofold) rabbit antisera prepared against the 34 recognized O-antigen strains and incubated at 4°C for 18 h. An isolate was considered to contain an O-antigen factor when at least 50% of the cells agglutinated in the serum at a dilution of 1/160 or less.

Isolation of bacteriophages which lyse *Yersinia*. Pooled raw sewage (100 ml per day for 5 days) was obtained from the Chapel Hill sewage treatment plant, Chapel Hill, N.C.; Shoals Creek sewage treatment plant, Atlanta, Ga.; and the Snapfinger sewage treatment plant, Decatur, Ga. About 2 to 4 ml of raw sewage was combined with 0.1 ml of an overnight TSB *Yersinia* culture in 9 ml of TSB. This mixture was incubated for 6 to 9 h. A 3-ml portion of the bacterium-sewage enrichment was transferred to a 13- by 100-mm screw-capped tube, and 0.3 ml of chloroform was added to kill the bacteria. The suspension was mixed vigorously on an orbital mixer (Vortex, model S8223; Scientific Products, Evanston, Ill.) and refrigerated at 4°C for at least 1 h to allow the chloroform to settle. One milliliter of the suspension was transferred to sterile multiwell plastic plates (Disposo trays, model FB16-24TC; Linbro Scientific Co., New Haven, Conn.) and placed in a laminar-flow safety cabinet which speeded the evaporation of residual chloroform.

A bacterial lawn was prepared by flooding a dry (30 min in a laminar-flow safety cabinet) TSA plate with an overnight TSB culture that had been adjusted to the standard turbidity. Alternatively, a tube of TSB/5 was inoculated from a TSA working culture and incubated until the turbidity equaled that of the standard. This usually occurred within about 16 to 18 h, and both methods produced confluent growth over the entire surface of the plate. All possible fluid was removed from the flooded plates with a sterile Pasteur pipette which was discarded into disinfectant (Amphyl; National Laboratories, Toledo, Ohio). The plates were dried at room temperature with the tops off for approximately 10 min.

The phage suspensions were diluted 10^{-2}, 10^{-4}, 10^{-6}, and 10^{-8} in TSB. With a sterile tuberculin syringe, 4 to 5 drops (about 0.01 ml each) of each dilution were placed on a TSA plate containing a lawn of the same bacterial strain that had been used to isolate the phage. The plates were kept at room temperature with the tops off until the drops had dried (about 20 min) and then were incubated for 16 to 18 h. After incubation, the plates were observed with background lighting in a model G100 colony counter (New Brunswick Scientific Co., New Brunswick, N.J.). Clear areas, or plaques, indicated lysis by a bacteriophage. An example of a phage titration is shown in Fig. 1.

Each phage isolated from raw sewage was purified by the soft-agar overlay method of Adams (1), modified as follows. A tube containing 3 ml of Oxoid Ionagar (0.4% agar) was melted and cooled to 50°C. From the 100-fold dilution series done previously, a volume was calculated that would yield 50 to 100 plaques. This volume was combined with 0.1 ml of an overnight host culture in the cooled agar, mixed gently on an orbital mixer, and overlaid onto a 13- by 100-mm TSA plate. After the agar had solidified (about 15 to 20 min), the plate was incubated overnight and observed for individual plaques. A well-isolated plaque was selected, and the tip of a sterile Pasteur pipette was stabbed through it to the bottom of the plate. The plaque was transferred to a tube containing 2.7 ml of TSB, and chloroform (0.3 ml) was added to kill the bacteria. The suspension was mixed well and refrigerated at 4°C for about 1 h. Two milliliters of the top

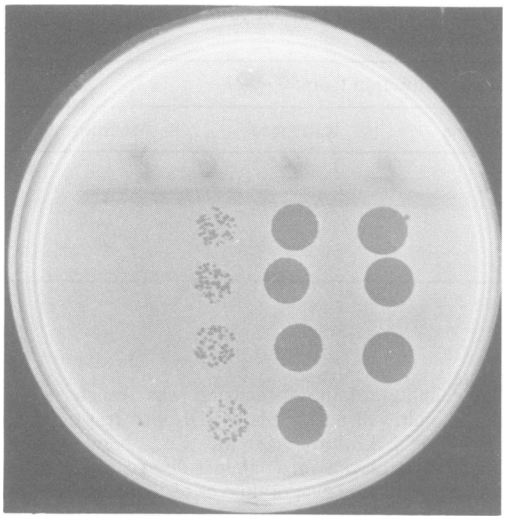

FIG. 1. Titration of bacteriophage K27 on *Y. enterocolitica* 9183-70; right to left: 10^{-2}, 10^{-4}, 10^{-6}, 10^{-8}.

layer was transferred to a sterile container which was placed uncovered in a laminar-flow safety cabinet to evaporate the residual chloroform. This suspension was titrated as previously described. The entire plaque-cloning procedure was done at least twice to ensure that the phage stock was derived from a single plaque.

The following procedure was used to prepare phage stocks with a high titer. One milliliter of the phage suspension obtained from the cloning procedure was mixed with 0.1 ml of an overnight TSB culture in 10 ml of TSB. A control growth tube was prepared by transferring 0.1 ml of the host strain alone to 10 ml of TSB. The test and control suspensions were incubated for 4 to 6 h or until the tube containing the phage cleared. The phage suspension was filtered through a 0.2-μm nitrocellulose filter (Nalgene Filter Unit, code PS, Nalgene Co., Rochester, N.Y.) to remove any unlysed bacteria or bacterial debris and titrated as previously described. All phages were grown to a titer of at least 10^8 PFU/ml. In some cases, this required a second step to give a hightiter. One milliliter of the first filtered suspension was transferred to 10 ml of TSB containing about 10^7 organisms. This second high-titer suspension was treated as before, and the final filtered stock was stored in sterile screw-capped tubes at 4°C.

Fifty bacteriophages were isolated. Two of these had to be discarded; phage K1 could not be adjusted to at least 10^8 PFU/ml, and K8 would not produce confluent lysis on its host strain at any concentration. The remaining 48 phages were isolated on the following *Yersinia* species: 19 on *Y. enterocolitica*, 13 on *Y. kristensenii*, 5 on *Y. frederiksenii*, and 11 on *Y. intermedia*.

Determination of the RTD. When phages are applied in very high concentrations, massive bacterial cell destruction can result without the production of new phage particles (1, 3). Concentrated phage suspensions are also more likely to contain a bacteriophage mixture (such as host range mutants or temperate phages). To minimize these problems, a routine test dilution (RTD) is used in most phage typing schemes. In many systems, the RTD is defined as the highest dilution that just produces confluent lysis or semiconfluent lysis on the propagating (host) strain (3). When this definition is used, the number of particles will vary with each phage because of differences in the size of the plaques. An alternative method is to define RTD as a specified number of phage particles or PFU. In this study, the RTD was defined as 10^6 PFU/ml. From tubes containing this number of phages, syringes that would dispense 0.01 ml per drop were filled. Since 1 drop was used for each test, the RTD was equal to 10^4 PFU per test (10^6 PFU/ml × 0.01 ml = 10^4 PFU in each).

Bacteriophage typing procedure. Typing was done on whole cultures (not single colonies), and incubation was at 36°C. Bacterial lawns were prepared as previously described, and the phages were dropped onto the lawns with the applicator (Johnny Brown Machine Shop, Tuscaloosa, Ala.) shown in Fig. 2. This applicator simultaneously delivers up to 60 drops, significantly speeding up the typing procedure. After the drops had dried for about 15 min at room temperature, the plates were incubated overnight and examined for lysis. The lytic reaction of each phage was recorded according to the definitions shown in Table 2. When possible, the actual number of plaques was counted. We arbitrarily defined 40 or more individual plaques (2+ lysis or greater) as a positive reaction and 39 or fewer individual plaques (1+ lysis or less) as a negative reaction. The reaction pattern of all the bacteriophages was called the lytic pattern. These lytic patterns were then converted into numbers with the notation shown in Table 3. This code was called the phage pattern. For example, if 12 reactions were + − − + − − − − + + +, the resulting code would be 5581. If the bacterial strain was not lysed by any of the phages, the code would be 8888.

Selection of bacteriophages. The bacteriophages that best differentiated the isolates within each *Yersinia* group were selected with the Phage-Cine computer program (developed by John Zakanycz and Milton Hutson, Computers Honors Program, University of Alabama [29]). The Phage-Cine computer program first selects the phage that best divides the isolates into two equal groups, with half (or closest to half) being lysed and half not being lysed. The second phage is selected that best divides each of these two groups into four more groups. The program continues to select phages that best divide the groups formed by the previous choices until the number of phages determined by the user has been reached.

A composite set of 24 phages was chosen by combining the best phages selected for each individual group. This composite set was evaluated for its ability to type bacterial strains belonging to the four groups and also to type strains from three outbreaks.

RESULTS

Antibiotic susceptibility. The susceptibilities of 305 isolates to 12 antibiotics are shown in Table 4. There were no appreciable differences in the susceptibility of the four *Yersinia* species to colistin, naladixic acid, sulfadiazine, gentami-

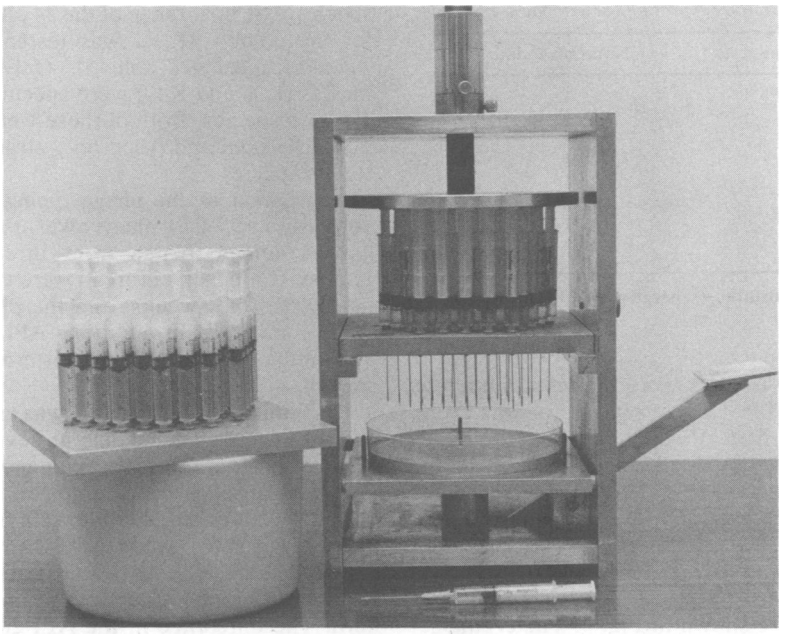

FIG. 2. Multisyringe applicator used to simultaneously deliver 1 drop of each bacteriophage.

cin, streptomycin, kanamycin, tetracycline, chloramphenicol, and penicillin. Strains of *Y. kristensenii*, however, had larger zones around cephalothin, ampicillin, and carbenicillin (Table 4). Only 11 (or 5%) *Y. enterocolitica* had zones of inhibition for carbenicillin of >25 mm, whereas the zones of all *Y. kristensenii* strains except one were >25 mm. When the zone sizes in millimeters around the disks for carbenicillin and ampicillin are plotted (Fig. 3), there is a clear differentiation of these two species. The larger zone around penicillin-cephalosporin antibiotics may provide an additional useful marker in differentiating *Y. enterocolitica* from *Y. kristensenii*.

Selection of bacteriophages. The number of phages selected to best differentiate isolates belonging to each *Yersinia* species were: *Y. enterocolitica*, 12; *Y. kristensenii*, 9; *Y. frederiksenii*, 12; and *Y. intermedia*, 12. These phages were combined into a composite set for typing all four *Yersinia* species (Table 5). Since *Y.*

TABLE 2. *Definitions of bacteriophage lysis used in this study*

Code for recording lysis	Reaction defined to be[a]	Definition of lysis[b]
CL	+	Confluent Lysis; completely clear zone of lysis with well defined edges
SC	+	Semi-Confluent lysis; less than confluent lysis; may contain some phage resistant colonies in zone; edges less well defined than in confluent lysis; may also include opaque lysis, in which clear zones of lysis are covered by a layer of apparently resistant bacteria
3+	+	80 or more individual plaques
2+	+	40 to 79 individual plaques
1+	-	10 to 39 individual plaques
-	-	9 or fewer individual plaques

[a] Used for converting the phage reaction into the notation code (Table 3).
[b] Adapted from Anderson and Williams (3).

TABLE 3. *Notation for reporting bacteriophage types (15)*

Results of three tests	Notation code
+++[a]	1
++−	2
+−+	3
−++	4
+−−	5
−+−	6
−−+	7
−−−	8

[a]Symbols: + = positive, − = negative (see Table 2 for definitions).

enterocolitica isolates were the most numerous (236 strains), the 12 best phages for this group were chosen first. Five phages for typing *Y. kristensenii* were added next. The remaining phages were added to the composite set in the order in which they had been selected for the individual set. Seven phages were added for *Y. frederiksenii* and *Y. intermedia*. Two examples of phage patterns with the 24 typing phages of the final set are shown in Fig. 4. The typing set divided the 301 strains into 105 different lysis patterns. No single phage pattern was shared by all four groups, although some patterns were common to two groups. For example, lysis pattern 5388 8888 was found in both *Y. enterocolitica* and *Y. kristensenii*, but was not a pattern found for *Y. frederiksenii* or *Y. intermedia*. The results of typing 301 isolates with the composite set of 24 phages are summarized in Table 6.

Host range of the bacteriophages. The host range of a phage is its ability to lyse strains other than the one on which it was isolated. A narrow host range is one in which only the same strain, or closely related strains, are lysed. A phage with a wide host range can lyse a variety of strains in the same species or even a different species. The host range of the 24 phages selected for the composite set was tested on the four *Yersinia* species (Table 5). Only two of the phages (K36 and K40) were specific for a single *Yersinia* species. Both of these were isolated on *Y. kristensenii* and lysed only strains belonging to this species.

Application of the phage typing system. The composite set of 24 phages was used to type *Y. enterocolitica* isolates from three outbreaks. Isolates from each outbreak were typed twice at an interval of 4 weeks, and the phage patterns were compared. In addition, API 20E profiles and antibiotic susceptibility patterns were determined.

(i) Outbreak 1. Outbreak 1 was an outbreak of diarrhea among chinchillas at several farms in California (Table 7). The epidemiological information furnished was incomplete, and only five isolates were tested. All five were from the same farm, but isolate 5 was received 10 months after the first four. Isolates 1 to 4 had the same O group, antibiotic susceptibility, and phage pattern. The difference in the API 20E profile for isolate 3 was due to a negative inositol reaction. This isolate was inositol positive, however, at 3 days by the conventional method. Although isolate 5 had the same O group and API 20E profile, the antibiotic susceptibility and phage patterns were very different from those of the other four isolates. This isolate had a much larger zone for carbenicillin (Table 7) and was lysed by only one phage. Based on the latter two results, isolate 5 was different from the first four, which also agrees with the epidemiological finding that it was separated in time by almost a year from the first four.

(ii) Outbreak 2. This 1974 outbreak (Table 8) occurred in a family living in Lee County, Kentucky (30). A 4-month-old girl (index case, iso-

TABLE 4. *Antibiotic susceptibilities of the four Yersinia species*

Antibiotic	Disk potency	Mean and standard deviation of inhibition zones for: *Y. enterocolitica* (240 strains)	*Y. kristensenii* (16 strains)	*Y. frederiksenii* (29 strains)	*Y. intermedia* (20 strains)
Colistin	10 μg	15 ± 3	17 ± 4	15 ± 4	16 ± 2
Naladixic Acid	30 μg	28 ± 5	36 ± 4	33 ± 6	29 ± 11
Sulfadiazine	250 μg	20 ± 6	21 ± 8	24 ± 7	27 ± 6
Gentamicin	10 μg	22 ± 4	29 ± 3	27 ± 5	27 ± 6
Streptomycin	10 μg	17 ± 4	20 ± 6	23 ± 5	21 ± 5
Kanamycin	30 μg	22 ± 5	28 ± 3	28 ± 6	27 ± 6
Tetracycline	30 μg	22 ± 5	29 ± 6	26 ± 5	27 ± 5
Chloramphenicol	30 μg	25 ± 6	29 ± 8	27 ± 8	29 ± 5
Penicillin	10 units	7 ± 2	8 ± 2	7 ± 2	8 ± 2
Ampicillin	10 μg	11 ± 4	20 ± 8	13 ± 6	15 ± 5
Carbenicillin	100 μg	15 ± 6	33 ± 9	18 ± 9	21 ± 7
Cephalothin	30 μg	11 ± 5	17 ± 8	11 ± 6	13 ± 5

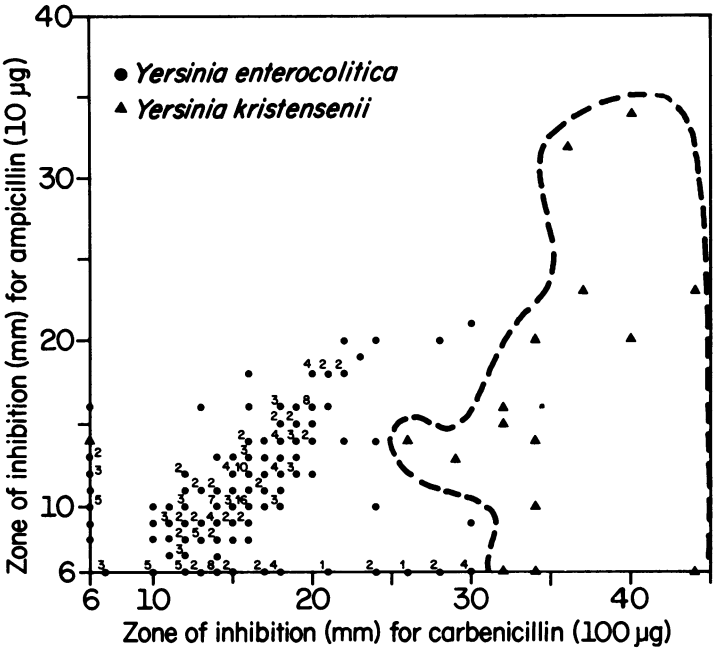

FIG. 3. Differentiation of *Y. enterocolitica* and *Y. kristensenii* by their zone sizes around ampicillin and carbenicillin disks. The number above and to the left of certain dots gives the number of strains with that particular result.

TABLE 5. *Host range of each bacteriophage (selected for the composite typing set) on the 4 species*

New phage designation	Old phage designation	Percent of isolates lysed:				
		Y. enterocolitica (236 strains)	*Y. kristensenii* (16 strains)	*Y. frederiksenii* (29 strains)	*Y. intermedia* (20 strains)	All 4 species (301 strains)
1	K29e[a]	50	25	10	10	43
2	K16e	27	13	7	10	23
3	K14e	23	0	10	10	20
4	K45s	21	50	17	5	21
5	K7e	19	13	3	10	17
6	K48e	19	6	0	0	15
7	K25s	10	6	10	5	9
8	K17i	8	0	45	70	16
9	K2s	3	25	10	5	5
10	K37i	6	0	24	20	9
11	K27e	25	0	10	0	21
12	K28e	26	0	7	5	21
13	K40s	0	31	0	0	2
14	K36s	0	19	0	0	1
15	K3s	1	13	24	25	5
16	K26f	1	13	17	10	4
17	K41e	1	19	0	0	2
18	K35i	2	0	24	55	7
19	K38i	0	6	3	20	2
20	K15i	0	0	45	70	9
21	K23i	1	0	3	10	1
22	K24s	2	13	62	15	9
23	K22i	1	0	45	15	6
24	K9e	63	0	10	10	23

[a] Lowercase letter represents the species on which the phage was isolated: e, *Y. enterocolitica;* s, *Y. kristensenii* (the s originally stood for sucrose negative); f, *Y. frederiksenii,* i, *Y. intermedia.*

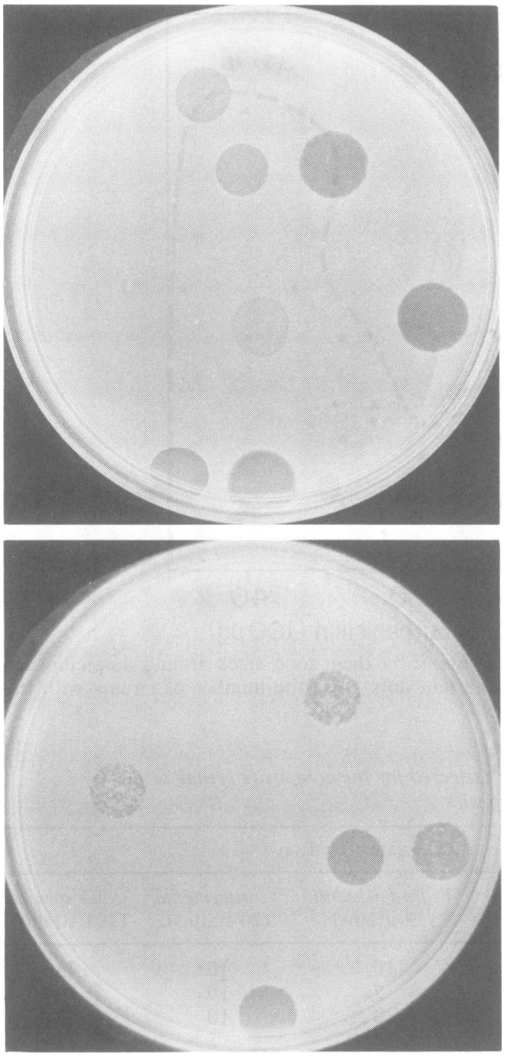

FIG. 4. Differentiation of two strains by their different lysis patterns.

late 1) had vaginal abscesses and lymph node swelling. There was no history of fever, vomiting, diarrhea, or vaginal discharges in the child or in 15 family members. An aspirate of one of the nodes yielded *Y. enterocolitica*, serogroup O 20. A month before the child's illness, the family's pet dog had given birth to 11 puppies. Eight of the puppies died of unknown causes. Stool specimens were collected from 10 family members and from the four remaining dogs. A sample of dried dog feces was also taken from the basement floor. Isolates 1 to 6 all had the same O serogroup, API 20E profile, antibiotic susceptibility pattern, and phage pattern. All six isolates had the same phage pattern at the second typing, but isolates 1 and 2 were 5688 8888 on the first typing and 8688 8888 on the second. All four typing methods indicate that isolates 1 to 6 are the same strain. This illustrates the importance of repeating a phage typing result which does not agree with other laboratory or epidemiological findings.

Isolates 7 to 10 had the same API 20E profile, but different O groups, antibiotic susceptibility patterns, and phage patterns. The phage patterns differed by at least two phage reactions at both typings. Serotyping and bacteriophage typing indicated that isolates 7 to 10 were different from the epidemic strain.

(iii) Outbreak 3. In September and October 1976, an outbreak (Table 9) of gastrointestinal illness occurred among school children in Oneida County, New York (9). The illness was characterized by fever and abdominal pain, and *Y. enterocolitica* serogroup O 8 was isolated from 38 ill children. Thirty-six had been hospitalized, and 13 had appendectomies because the symptoms mimicked appendicitis. In nine of the cases in which appendectomies had been performed, however, the appendix was normal or only slightly inflamed. The cause of the outbreak was finally traced to *Y. enterocolitica* which had contaminated chocolate milk that had been distributed as part of the school lunch program. Isolates 1 to 8 were epidemiologically implicated by case histories and had identical serogroups, antibiotic susceptibility patterns, and phage patterns (Table 9). When typed a second time, the phage patterns were the same. The only differences were in the API 20E profiles which had negative Voges-Proskauer reactions for isolates 6 and 8. They were also negative by the conventional method at 48 h. It is interesting to note

TABLE 6. *Ability of the typing phages to differentiate strains of each species*

Species	Number of strains studied	Number of different phage patterns	Percent of isolates in most common phage pattern	Percent of isolates lysed
Y. enterocolitica	236	66	9%	92
Y. kristensenii	16	14	13%	100
Y. frederiksenii	29	22	17%	97
Y. intermedia	20	14	15%	90

TABLE 7. *Typing results for Y. enterocolitica isolated from outbreak 1*

Isolate	Enteric Section's Number	Source	O-serogroup	API profile	Zone of inhibition (mm) for[a] AM	CB	CF	Phage pattern
1	9341-78	Chinchilla	3	1 114 723	6	8	6	3784 8887
2	9342-78	Chinchilla	3	1 114 723	6	11	6	3784 8887
3	9343-78	Chinchilla	3	1 114 523	6	10	6	3784 8887
4	9344-78	Chinchilla	3	1 114 723	6	10	6	3784 8887
5	9346-78	Chinchilla	3	1 114 723	6	34	6	5888 8888

[a]AM, ampicillin; CB, carbenicillin; CF, cephalothin
[b]Isolate No. 5 received ten months after first four.

that a strain isolated in 1932 (isolate 9) was nearly identical to the epidemic strain by all typing methods. This could be due to chance, or it could indicate a continuous reservoir of this strain. Isolates 10 to 21 were obtained from various sources in Oneida and surrounding counties but were not implicated in the outbreak. Bacteriophage typing indicated that these isolates were different from the epidemic strain even though five were also serogroup O 8. The zone size around the cephalothin disk was also a useful marker; the epidemic strain was susceptible, but 9 of the 13 other strains were resistant.

DISCUSSION

In the United States, biotyping and serotyping have been used to differentiate strains of *Y. enterocolitica* involved in outbreaks. Unfortunately, most of the American isolates involved in human infections have been serogroup O 8, which has greatly reduced the value of serotyping in epidemiological analysis. More sensitive methods for differentiating strains within this serogroup have been needed, but have not been available. No typing methods have been specifically designed for *Y. kristensenii, Y. frederiksenii*, and *Y. intermedia* because they have only recently been recognized as new species. Some strains in these three new species may be typable, however, with methods designed for *Y. enterocolitica*.

Biotyping for epidemiological analysis involves the use of biochemical tests to differentiate an epidemic strain from similar strains that may come from a variety of sources. These procedures are usually within the capability of most laboratories, since biochemical tests are widely used to identify bacteria. However, there is much laboratory-to-laboratory variation in the methods used, so biotyping results may not be consistent from one laboratory to another. Variables in biotyping include the type or lot number of medium used, inoculum size, incubation temperature and time, reagents, and definition of a positive reaction. A comparison of the API 20E system with the conventional biochemicals used to define the four *Yersinia* groups indicated that isolates identified as *Y. enterocolitica* by the API 20E system may require further testing with

TABLE 8. *Typing results for Y. enterocolitica isolated from outbreak 2*

Isolate	Enteric Section's Number	Source	O-serogroup	API profile	Zone of inhibition (mm) for[a] AM	CB	CF	Phage pattern
1	9286-78	Human (index case)	20	1 154 523	16	18	16	8688 8888
2	9287-78	Dog #3 (feces)	20	1 154 523	19	19	18	8688 8888
3	9288-78	Dog #4 (feces)	20	1 154 523	21	20	20	8688 8888
4	9289-78	Dog #1 (feces)	20	1 154 523	17	22	16	8688 8888
5	9290-78	Dog #2 (feces)	20	1 154 523	14	20	16	8688 8888
6	9148-78	Feces (basement floor)	20	1 154 523	16	17	19	8688 8888
7	9291-78	Human (cousin)	6,30	1 154 523	6	15	6	5888 8888
8	9292-78	Human (Aunt)	NT[b]	1 154 523	22	40	20	5558 8888
9	9293-78	Human (Grandfather)	NT	1 154 523	20	22	18	5388 8888
10	9294-78	Human (cousin)	NT	1 154 523	20	28	19	8858 8888

[a]AM = ampicillin, CB = carbenicillin, CF = cephalothin
[b]NT = nontypable

TABLE 9. *Typing results for Y. enterocolitica isolated from outbreak 3*

Isolate	Enteric Section's Number	Source	O-Serogroup	API profile	Zone of inhibition for[a] AM	CB	CF	Phage pattern
			Epidemiologically implicated					
1	9127-79	Human	8	0 155 523	16	19	19	6858 8888
2	9128-79	Human	8	0 155 523	18	20	18	6858 8888
3	9129-79	Human	8	0 155 523	16	20	18	6858 8888
4	9130-79	Human	8	0 155 523	16	18	18	6858 8888
5	9131-79	Human	8	0 155 523	16	20	19	6858 8888
6	9132-79	Human	8	0 154 523	17	18	18	6858 8888
7	9133-79	Human	8	0 155 523	17	20	18	6858 8888
8	9149-79	Chocolate milk	8	0 154 523	16	18	18	6858 8888
			Not epidemiologically implicated					
9	9134-79	1932 isolate	8	0 154 723	16	20	21	6858 8888
10	9135-79	Cow (cervix)	8	0 154 523	14	16	16	8688 8888
11	9136-79	Milk F1157170	8	0 154 723	10	13	6	8888 8888
12	9137-79	Cow (feces)	8	0 154 723	12	16	6	8888 8888
13	9138-79	Raw milk	8	0 154 723	12	14	6	8888 8888
14	9139-79	Cow (feces)	8	0 154 523	11	14	6	8888 8888
15	9140-79	Cow (feces)	4,33	0 154 523	10	15	6	8888 8888
16	9141-79	Human	4,33	0 155 723	11	14	12	8888 8888
17	9142-79	Human	6,31	0 154 723	12	14	6	5588 8888
18	9143-79	Cow (feces)	6,31	0 154 723	10	14	6	6888 8888
19	9144-79	Milk	6,31	0 154 723	10	11	6	6888 8888
20	9145-79	Milk	6,31	0 154 723	10	12	6	6888 8888

[a] AM = ampicillin, CB = carbenicillin, CF = cephalothin.

conventional biochemical methods for detecting acid production in L-rhamnose, raffinose, and melibiose. This may be necessary to detect *Y. intermedia* and *Y. frederiksenii*.

Antiobiograms were determined for 305 *Yersinia* isolates. Most strains were susceptible to colistin, naladixic acid, sulfadiazine, gentamicin, streptomycin, kanamycin, tetracycline, and chloramphenicol, but resistant to penicillin. *Y. kristensenii* was more sensitive than *Y. enterocolitica* to ampicillin, carbenicillin, and cephalothin. In addition to providing an additional marker for recognizing this species, the data lend further support to the proposal that *Y. enterocolitica* and *Y. kristensenii* should be considered as separate species. No differences were noted in the susceptibilities of the other *Yersinia* groups. In our epidemiological studies, antibiotic susceptibility patterns were useful in several instances, but differences should be interpreted with caution because they could be due to the selective pressures of antibiotic usage.

The new bacteriophage typing system appears useful in differentiating strains in each of the four *Yersinia* species. Forty-eight phages that had been isolated from raw sewage were tested against 301 isolates. The best phages for differentiating each group were selected by computer, and these phages were combined into a composite typing set of 24 phages. Some of the criteria for a practical bacteriophage typing system have been outlined by Anderson and Williams (3) as follows: (i) the typing system should divide the isolates into a sufficient number of types; (ii) the typing method should be simple and give clear-cut results; (iii) the phage types should be stable and reproducible; (iv) the typing results should agree with epidemiological information; and (v) the typing method should be well standardized before being accepted as a tool in epidemiological analysis.

With the composite set of 24 phages, isolates from the four *Yersinia* species could be divided into 106 different phage patterns. Only 10 (9%) of the phage patterns were found in more than one *Yersinia* species. This new set has a marked improvement for differentiation over the phage typing systems developed in Europe. For example, in the typing method reported by Mollaret and Nicolle (24), one phage pattern accounted for 57% of the isolates, and in the typing systems of Nilehn and Ericson (27), one type accounted for 87% of the human isolates.

A critical factor in any typing system is how consistently the results can be read and interpreted. Simplified procedures have been pro-

posed for preparing host lawns and applying phages. Consistent results also depended upon the endpoints used to define phage lysis. An RTD of 10^4 PFU per test was used in this study, and the presence of 40 or more individual plaques was considered a positive reaction. This usually produced clear-cut areas of lysis that were easy to read, but there were exceptions. For example, when three cultures of the same *Y. kristensenii* strain were typed, phage K45 produced 3, 36, and 42 plaques on the three plates. With the definitions used in this study, the first two would be called negative, and the third would be called positive. However, from a practical standpoint, there is no difference between 36 and 42 plaques. The plate with three plaques is harder to explain, but may have been due to differences in the titer of the phage suspension applied to the host lawn or to other slight variables in the typing method. When lower-endpoint definitions (fewer plaques) were tried (i.e., 20 or more individual plaques considered positive), the problem still existed; it was only shifted down to the lower endpoints. This points out the need for some flexibility in interpreting the endpoints of phage typing systems. The best method is to visually compare the plates of all strains being compared. Slight differences in lysis patterns should not be weighed too heavily.

The third criterion of Anderson and Williams (13) is that the phage patterns, or types, should be stable and reproducible. There were no significant differences in the phage patterns for *Y. enterocolitica* isolates when these strains were all typed at the same time, but when the same strains were typed again after 4 weeks, only 90% of the isolates had the same pattern as at the first typing. Ninety-eight percent of *Y. kristensenii* and 93% of the *Y. frederiksenii* isolates had identical phage patterns when typed simultaneously, but only 40 and 50%, respectively, when typed at different times. Ninety-two percent of the *Y. intermedia* strains could be correctly identified when typed at the same time, but only 40% of the strains had exactly the same pattern 4 weeks later. Many factors can affect the reproducibility of a typing method. Different lots of media, variations in preparing host lawns, different methods for applying phage suspensions, and even changes in phage titer can cause changes in the amount of lysis. Because a certain amount of variability is expected in any biological system, many phage typing systems allow "identical strains" to differ by as many as two or three phage reactions (3). In this study, 11 of the changes in phage patterns were due to differences in only one phage. Seven changes in phage pattern were due to differences in two phages, and two phage patterns changed by more than two phage reactions. If the two-phage difference were applied to the data in this study, all of the *Y. enterocolitica* and *Y. kristensenii* and 90% of the *Y. frederiksenii* and *Y. intermedia* isolates could be correctly identified on the basis of their phage patterns. Isolates from the three outbreaks were typed twice at an interval of 4 weeks. Except for two isolates in outbreak 3, all of the phage patterns were identical at both typings. These two isolates had patterns of 5688 8888 at the first typing and 8688 8888 at the second. To eliminate the variable of time, we strongly recommend that all isolates being compared should be typed on the same day.

Probably the most important aspect of a phage typing system is the agreement with epidemiological findings and other laboratory typing methods. In this study there were interesting correlations among phage typing, other laboratory methods, and epidemiological information. For example, in outbreak 1, both the antibiogram and the phage pattern differentiated the first four epidemic strains from an isolate received 10 months later, even though the serogroups were the same. In outbreak 3, phage typing also differentiated all but one of the isolates which were unrelated to the immediate outbreak. This outbreak illustrates the usefulness of bacteriophage typing in differentiating strains of serogroup O 8.

All typing methods should be carefully standardized. Several precautions can ensure the effective use of the typing system developed in this study. First, the same methods should be used for typing all cultures. The use of the same lot of media, standardized inoculum, and uniform methods of preparing host lawns and applying phages was an attempt to meet this criterion. Second, all of the isolates obtained from an outbreak should be typed at the same time. Differences in lytic patterns should be interpreted with caution if the typings are done at different times. Third, the phage reactions on different strains should be compared visually to give some flexibility in reading at or near the endpoint of a positive reaction. Fourth, quality control strains should be included with each typing run to check for changes in phage titer. We concluded that most of the "changes" in lytic patterns were really due to variability in reading weak reactions.

The results of this study indicate that the new phage typing system can be helpful in the epidemiological analysis of outbreaks due to the four *Yersinia* species. It can also be used to differentiate strains that belong to the same O group. Further research should focus on a number of different areas. Outbreaks due to *Y. kristensenii*, *Y. frederiksenii*, and *Y. intermedia* should be studied to test the efficacy of the typing system on these new groups. New phages could be

isolated that further differentiate strains with common lysis patterns. The quality control procedures could be simplified if there was a single bacterial strain lysed by all the phages. Although bacteriophage typing is generally more complicated than either biotyping or antibiotic susceptibility testing, all of the methods described here can be done in most well-equipped laboratories. However, phage typing, along with serotyping, may be more appropriate for reference laboratories. We encourage others to try the typing phages we have used; these may be obtained by contacting P.M.B. at his present address.

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