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Evaluation of Disk Method for Hippurate Hydrolysis by *Campylobacter* Species

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A disk method for hippurate hydrolysis was compared with the ninhydrin tube method by using 140 genetically confirmed *Campylobacter* strains. Results were similar for 129 (92%) strains when the inoculum size for the disk method was standardized. Six strains (4.2%) showed variable results by each method. Our results conflict with those obtained in studies by others, who found the two methods to be dissimilar.

Hippurate hydrolysis is an important phenotypic test used in the identification of *Campylobacter* species that cause human illness. The rapid tube hippurate test, first described by Hwang and Ederer (6) and modified for use with *Campylobacter* spp. by Harvey (5), is frequently used to distinguish *Campylobacter jejuni* from other thermophilic *Campylobacter* species, namely, *C. coli* and *C. lari*. Most *C. jejuni* strains hydrolyze hippurate to glycine and benzoic acid; other *Campylobacter* species and a small percentage of *C. jejuni* strains lack this trait (14).

Two modifications of the rapid hippurate tube test that use paper disks impregnated with 1% sodium hippurate have been applied to campylobacters (4, 8). Phenotypically identified *Campylobacter* strains from human, nonhuman, and poultry sources were used as test strains in both evaluations. Kotsis and Adam (8) compared their disk method with the modified tube method of Skirrow and Benjamin (13), using 169 *C. jejuni* and *C. coli* strains. Results obtained by the two methods were comparable. Cacho et al. (4) compared the disk method (Remel, Lenexa, Kan.) and the ninhydrin tube test used at the Centers for Disease Control and Prevention (CDC), Atlanta, Ga. (9), with a high-performance liquid chromatography (HPLC) procedure for hippurate hydrolysis (2, 15). The disk method showed 93% sensitivity and 94% specificity, whereas the CDC method showed 80% sensitivity and 81% specificity when test results for 118 *Campylobacter* strains were compared with those obtained by the HPLC method.

We compared the Remel disk method with the CDC tube method using 140 *Campylobacter* strains that were characterized by DNA-DNA hybridization (3) and/or multilocus enzyme electrophoresis (MEE) (1, 10, 12). DNA-DNA hybridization identifies a strain to species level at the more stable genetic level rather than by phenotypic characteristics. In DNA-DNA hybridization studies, our test strains showed 55% or higher DNA relatedness to the *Campylobacter* type strain at the stringent reassociation temperature (65°C), thereby fitting the species definition (3). MEE detects chromosomal differences in a cell that affect the electrophoretic charge on the protein. This isoenzyme electrophoretic technique generates electrophoretic types that are used to study genetic relatedness among bacterial species. Electrophoretic types determined for strains of *Escherichia coli* and *Salmonella typhi* and nine *Campylobacter* species correlated well with results of DNA hybridization tests (10–12).

The sources and DNA-DNA hybridization and MEE identification test results for the 140 test strains are listed in Table 1. The coded strains were grown on heart infusion agar with 5% rabbit blood at 36°C for 48 h in a microaerobic atmosphere of approximately 5% O_2, 7.5% CO_2, 7.5% H_2, and 80% N_2 and were tested at least twice by the disk (manufacturer's protocol) and the CDC tube methods (9). The hippurate-negative *C. jejuni*, the *C. coli* strains, and those strains that gave inconsistent readings in two consecutive tests were tested for hippuricase activity by using a gas-liquid chromatograph (GLC) equipped with a thermal conductivity detector, according to the procedure of Kodaka et al. (7). Positive and negative control strains (*C. jejuni* ATCC 33560 and *C. coli* ATCC 33559) were included with each testing of the three methods.

In preliminary studies, the amount of inoculum (one to three colonies) for the disk method described by the manufacturer (Remel) was sometimes difficult to ascertain because *Campylobacter* growth is often confluent rather than in distinct colonies. Also, hippurate-negative strains appeared to be hippurate positive because of the development of a light purple color (the manufacturer defined hippurate-negative strains as clear or light gray). Therefore, we used genetically confirmed *C. jejuni* and *C. coli* strains to standardize the inoculum size and to establish the color range for a hippurate-negative test result. The inoculum was changed to that quantity of bacteria needed to fill a 1-μl plastic loop (Nunc, Roskilde, Denmark). The inoculum (48-h culture) was emulsified in a plastic tube containing 0.1 ml of sterile distilled water. A hippurate disk (Remel) was added, and the mixture was incubated for 2 h at 37°C. Two drops of ninhydrin reagent were added, and the mixture was reincubated for 20 to 30 min. A positive test result was shown by a purple to dark blue color (as described by the manufacturer of the disk), but strains testing negative were clear, light gray, or light to medium purple. To interpret the borderline (medium purple) color, the end product color of the test strain was compared with that of the negative control strain (ATCC 33559), for which a test was run simultaneously.

For the tube method, the previously described CDC procedure was used (9). A 10-μl loopful (Nunc) of 48-h bacterial growth was emulsified in 0.4 ml of 1% sodium hippurate and incubated for 2 h at 37°C. The emulsion was overlaid with 0.2 ml of 3.5% ninhydrin in a 1:1 mixture of acetone and butanol

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TABLE 1. Sources and genetic confirmations of 140 *Campylobacter* test strains

Campylobacter species[a]	No. of strains	Source[b]	No. of strains confirmed by:	
			DNA[c]	MEE
C. jejuni subsp. *jejuni*, hipp.+	27	Human	27	27
	6	Animal	6	6
	3	Fowl	3	3
	1	Unknown	1	1
C. jejuni subsp. *jejuni*, hipp.−	6	Human	4	6
	5	Fowl	5	5
C. jejuni subsp. *jejuni*, Nal^r	9	Human	9	9
C. jejuni subsp. *doylei*	9	Human	1	9
C. coli	26	Human	13	22[d]
	7	Animal	7	7
	4	Fowl	4	4
	2	Unknown	1	2
C. lari	22	Human	18	22
	1	Animal	0	1
	11	Fowl	9	11
	1	Water	1	1
Total	140			

[a] hipp.+, hippurate hydrolysis positive; hipp.−, hippurate hydrolysis negative; Nal^r, nalidixic acid resistant.
[b] Animal (domestic) sources were cows, sheep, goats, and pigs. Fowl sources were wild birds or chickens.
[c] DNA, DNA-DNA hybridization.
[d] Four strains not identified by MEE were identified by DNA-DNA hybridization.

and reincubated for 10 min at 37°C. A deep purple color indicated a positive result. Weaker shades of purple indicated a negative result.

Of the 140 test strains, 129 gave comparable reactions by the disk and tube methods. One *C. jejuni* strain (D127) was hippurate negative by the disk and tube methods and hippurate positive by GLC, whereas 10 strains gave variable results by one or both methods and were tested a third time (Table 2).

TABLE 2. Variable hippurate hydrolysis reactions in *Campylobacter* strains[a]

Species and strain	Result by methods[b]:		
	Tube	Disk	GLC
C. jejuni D127	−, −	−, −	+
C. jejuni D117	+, −, +	+, +, +	+
C. jejuni D125	+, −, +	+, +	+
C. jejuni D128	±, +, ±[c]	−, +, +	+
C. jejuni D2627, Nal^{rc}	+, −, +	+, +	+
C. jejuni D977, hippurate negative	−, −, −	+, −, −	−
C. jejuni subsp. *doylei* D2295	+, −, +	+, −, +	+
C. jejuni subsp. *doylei* D2722	+, +, +	−, +, +	+
C. coli D1038	−, −, +	+, −, +	−
C. lari D71	+, −, −	−, −, −	−
C. lari D459	−, −, −	+, −, −	−

[a] Strains were tested two or more times by the tube and disk methods.
[b] Tube, ninhydrin tube test; disk, Remel disk test; GLC, gas-liquid chromatography. +, positive reaction; −, negative reaction; ±, borderline reaction.
[c] Nal^r, nalidixic acid resistant (30 μg) (disk method).

These results were confirmed as either positive or negative by the more sensitive and reproducible GLC method. The situation with *C. coli* D1038 exemplifies the difficulties with test-to-test variability and interpretation in the disk and tube procedures with some strains; false-positive results were obtained one or more times by both methods. Variable reactions in the tube test have been previously reported for this strain (9). Overall, the tube and disk methods were comparable, i.e., six strains (4.2%) showed variable results by each procedure. The reliabilities of the two methods (using the first testing result) for species identification were 97.8% for the tube test and 95.7% for the disk method (Table 2). Misidentification (using the first testing result) would have occurred with two *C. jejuni* strains and one *C. lari* strain by the tube test and with three *C. jejuni* strains, one *C. jejuni* subsp. *doylei* strain, one *C. coli* strain, and one *C. lari* strain by the disk test.

Our data are inconsistent with work by Cacho et al. (4), who found the Remel disk test more sensitive than the CDC tube test. Both procedures depend on the reaction of ninhydrin and glycine to yield a purple compound that may vary in shade and intensity in strains with low levels of hippuricase and with nonspecific reactivity of ninhydrin with primary amines and peptides in the test medium (15). Cacho et al. and others found the ninhydrin tube test difficult to interpret because of the varying color density of the end product (4, 9, 15). We found the disk method to be equally difficult to interpret and standardized the inoculum to 1 μl, which produced better color correlation between duplicate tests and diminished a potential source of false-positive readings.

No modifications to the CDC tube procedure were made (9). The discrepancy between the results obtained in our study and in that of Cacho et al. may be attributed in part to variability in interpretation of results by the two laboratories, nonstandardization of the inoculum for the disk method, and use of test strains that were identified to the species level by phenotypic tests. Thermophilic *Campylobacter* spp. may occasionally be misclassified when species identification is based on a single phenotypic trait such as the hydrolysis of hippurate. False-negative results by the tube procedure were obtained for 3 of 41 (7.3%) thermophilic *Campylobacter* strains identified by DNA homology (9). Nonstandardization of the disk test inoculum resulted in hippurate-negative strains appearing hippurate positive. Studies show that the sensitivities of the CDC procedure and other modifications of the Hwang and Ederer (6) ninhydrin procedure are comparable but that all these methods are less sensitive than the GLC methods (2, 7, 9, 15). Thus, we agree with others that thermophilic *Campylobacter* strains with weak or questionable hippuricase activity as determined by the ninhydrin procedure (disk or tube) should be confirmed by GLC (7, 9).

In summary, we found the Remel disk with a standardized inoculum and the CDC tube method for hippurate hydrolysis to be comparable. The development of a medium purple pigment by some hippurate-negative *Campylobacter* strains was equally difficult to interpret by both methods. The disk method was simple to use and required fewer cells than the tube method did. This is useful when a strain grows sparsely on the agar plate. Reagents for the disk method are available commercially and may be cost effective for laboratories that occasionally need to identify *Campylobacter* species and do not choose to prepare the test reagents.

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