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Comparison of Four Hippurate Hydrolysis Methods for Identification of Thermophilic *Campylobacter* spp.

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The test for hippurate hydrolysis is critical for separation of *Campylobacter jejuni* and *C. coli* strains. Glycine and benzoic acid are formed when hippurate is hydrolyzed by *C. jejuni*. The test used in most laboratories is one of several variations of the ninhydrin tube test described by Hwang and Ederer (M. Hwang and G. M. Ederer, J. Clin. Microbiol. 1:114–115, 1975) for detection of glycine. We evaluated three modifications of the Hwang and Ederer method and the gas-liquid chromatographic (GLC) method described by Kodaka et al. (H. Kodaka, G. L. Lombard, and V. R. Dowell, Jr., J. Clin. Microbiol. 16:962–964, 1982) for detecting benzoic acid. *Campylobacter* strains comprised 22 *C. jejuni*, 11 *C. coli*, and 8 *C. laridis* strains. The species identification of each strain was confirmed by DNA relatedness. All strains of *C. jejuni* were positive and all strains of *C. coli* and *C. laridis* were negative by the GLC method for detecting hippurate hydrolysis, whereas three strains of *C. jejuni* gave negative or variable results in the tube tests. The GLC method is more sensitive than the tube methods for detecting hippurate hydrolysis and should be used on cultures yielding variable or questionable test results.

It is generally agreed that *Campylobacter jejuni* is an important cause of diarrhea in humans. Two other species of thermophilic *Campylobacter*, *C. coli* and *C. laridis* (nalidixic acid-resistant thermophilic *Campylobacter*) (1, 14), have also been implicated. Other bacteria frequently implicated in diarrheal diseases, such as members of the families *Enterobacteriaceae* and *Vibrionaceae*, can be definitively characterized by a large number of biochemical tests. In contrast, very few useful tests for distinguishing species in the genus *Campylobacter* are available. As a result, a few tests assume critical importance for taxonomic work with this genus, and the hippurate hydrolysis test is valuable for distinguishing *C. jejuni* from other species of *Campylobacter*, especially the other thermophilic species *C. coli* and *C. laridis* (6, 12, 15). Unlike the other species, *C. jejuni* usually hydrolyzes hippurate to benzoic acid and glycine. Several tube tests for hippurate hydrolysis have been described for *Campylobacter* spp. The tube tests are simple to conduct but sometimes present difficulties in interpretation. The gas-liquid chromatography (GLC) procedure (8) is technically more difficult and requires special equipment, but is described as more reproducible than the conventional tube methods. Because of the importance of hippurate hydrolysis and reports of variations in results among laboratories, we evaluated three tube methods used in many laboratories and compared these with the GLC procedure. The methods evaluated were the rapid 2-h tube procedure of Hwang and Ederer (7), described for *Campylobacter* spp. by Harvey (4), which we refer to as the Harvey method; the slow 4-h tube procedure of Skirrow and Benjamin (13), which we refer to as the Skirrow method; a modification of the method of Hwang and Ederer routinely used at the Centers for Disease Control (CDC) (10), described below, which we refer to as the CDC method; and the procedure of Kodaka et al. (8), which we refer to as the GLC method.

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MATERIALS AND METHODS

Strains. A total of 41 strains of *Campylobacter*, comprising 22 strains of *C. jejuni*, 11 strains of *C. coli*, and 8 strains of *C. laridis*, were used in this study. For all procedures the strains were grown on blood agar plates at 36°C in an atmosphere of 5% O_2–10% CO_2–85% N_2. They were phenotypically characterized by methods described previously (10).

Hippurate procedures. Three tube tests were used for testing hippurate hydrolysis; all three were modifications of the Hwang and Ederer method (7). The salient points of each method are described in Table 1. The CDC method (10) was conducted as follows. A loopful of an overnight growth on heart infusion agar containing 5% defibrinated rabbit blood was emulsified in 0.4 ml of 1% aqueous sodium hippurate. The dense suspension was incubated in a 37°C water bath for 2 h. After incubation, 0.2 ml of ninhydrin solution (3.5% ninhydrin in a 1:1 mixture of acetone and butanol) was slowly added with a pipette down the side of the tube to form an overlay. After incubation for a further 10 min in the 37°C water bath, the tube was examined for the appearance of a deep purple color (crystal violet). The Harvey method was similar to the CDC method, but required a large loopful of inoculum from a 48-h culture grown on thioglycolate agar with 5% sheep blood; after the ninhydrin solution was added, the suspension was incubated in air at 37°C. After we initiated this study, Harvey and Greenwood (5) modified the hippurate procedure of Harvey (4) and used 24- to 48-h growth from brucella agar with 5% sheep blood. The Skirrow method differed from the rapid methods (Harvey and CDC) in that the reaction time for ninhydrin was 2 h. The ratio of sodium hippurate to ninhydrin was 2.5:1; the amount of inoculum was suspended to an optical density of 1.0 at 540 nm; and the medium for growth of the strains was blood agar base no. 2 (Oxoid Ltd., London, England) with 5% horse blood. For tabulation of all tube methods, only the development of a deep purple color was considered positive, be-

TABLE 1. Description of four hippurate hydrolysis methods

Method	Medium	Incubation conditions	Age of culture for inoculum	Amt of inoculum	Amt of reagent (sodium hippurate: ninhydrin)	Ninhydrin addition; incubation time	Interpretation of results:	
							Positive	Negative
Harvey	Thioglycolate agar + 5% sheep blood[a]	GasPak without catalyst[b] 5% O_2	48 h	Large loopful	2:1	Overlay; 10 min, 37°C	Deep purple (crystal violet)	No color or faint purple
CDC	Heart infusion agar + 5% rabbit blood	5% O_2–10% CO_2–85% N_2	18–24 h	Loopful	2:1	Overlay; 10 min, 37°C	Deep purple crystal violet	Colorless or light purple
Skirrow	Oxoid blood agar base no. 2 + 5% horse blood	5% O_2–10% CO_2–85% N_2	Overnight	2 ml suspended to OD[c] of 1.0	2.5:1	Overlay; 2 h, 37°C	Deep purple	No color or pale mauve
GLC	CDC anaerobe blood agar	5% O_2–10% CO_2–85% N_2	48–72 h	0.2-ml suspension diluted to McFarland no. 3	NA[d] (HFF broth, 36h)	NA[d] (methanol-chloroform extraction)	Benzoic acid detected by GLC	No benzoic acid

[a] Harvey added 5% sheep blood or 7% human blood (S. M. Harvey, personal communication).
[b] We used 5% O_2–10% CO_2–85% N_2 instead of GasPak jar and GasPak generator without catalyst.
[c] OD, Optical density.
[d] NA, Not applicable.

cause *C. laridis* and *C. coli* sometimes gave weak to medium purple color reactions. Occasionally, strains showed various shades of purple in layers with the Skirrow method. Strains with a deep purple in one of the layers were considered positive. A positive reaction in the above tube tests indicates the formation of glycine as hippurate is hydrolyzed by the bacterium.

All GLC analyses for hippurate hydrolysis were done on a Gow Mac Chromatograph (model 550) equipped with a thermal conductivity detector as described by Lombard and Dowell (9) after the strains were grown in hippurate-formate-fumarate (HFF) broth (8). The inoculum suspension for the HFF broth was adjusted to match the turbidity of a McFarland no. 3 standard from 48- to 72-h growth on CDC anaerobe blood agar (3). To detect benzoic acid, an end product of hippurate hydrolysis, we mixed 1 ml of a 36-h HFF broth culture with 0.4 ml of 50% (vol/vol) aqueous H_2SO_4 and 2 ml of methanol in a screw cap tube (16 by 100 mm) and incubated the mixture in a 50°C water bath overnight. Then 1 ml of distilled water and 0.5 ml of chloroform were added to the tube, which was inverted about 20 times. The tube was then centrifuged for 5 min (1,500 to 2,000 rpm) to break any emulsion and aid in separating the chloroform layer from the aqueous portion. Chloroform extract (14 μl) was injected into a column packed with 10% SP-1000–1% H_3PO_4 on 100/120 Chromosorb WAW (Supelco Inc., Bellefonte, Pa.). The conditions under which the chromatograph was used for the analysis of the methylated acids were as follows: column temperature, 135°C; injection port temperature, 200°C; detector temperature, 185°C; carrier gas, helium; carrier gas flow rate, 120 ml/min; attenuation, ×2. A positive result was defined as a benzoic acid peak equal to or greater than the control peak produced by a solution of 0.122 g of benzoic acid in 100 ml of distilled water. A weak positive result was defined as a benzoic acid peak less than the 1-mM benzoic acid control but higher than the small benzoic acid peak from an uninoculated tube of HFF broth. A negative result was defined as a benzoic acid peak no greater than the benzoic acid peak in an uninoculated tube of HFF broth.

The strains were coded and tested on the same day by the three tube methods. The strains were also coded for testing by the GLC method but were not always tested on the same day as that for the tube methods. Group A and B streptococci were used as negative and positive controls.

Reproducibility of the tube methods was determined by retesting 20 strains by all three tube methods. Isolates selected for reproducibility were five strains positive by all methods, three strains with more than one color in the layers by the Skirrow method, three strains with different results by more than one method, and nine negative strains with weak or medium purple results. All 41 strains were tested for a second time by the GLC method.

DNA relatedness. DNA was prepared from 41 strains of *Campylobacter*. The techniques used in the isolation and purification of DNA and those used in determining DNA relatedness by the hydroxyapatite method have been described previously (2). DNAs from the type strains of *C. jejuni*, *C. coli*, and *C. laridis* were labeled in vitro with ^{32}P-labeled dCTP provided in a Nick Translation Kit (no. 8160SB; Bethesda Research Laboratories, Inc., Gaithersburg, Md.). Labeling was done as specified by the manufacturer. Labeled DNA was purified by passage over a Bio-Gel P-100 column (Bio-Rad Laboratories, Richmond, Calif.) equilibrated in 0.015 M NaCl–0.0015 M sodium citrate.

RESULTS

The *Campylobacter* strains were tentatively grouped on the basis of biochemical reactions, and their identity was confirmed on the basis of relatedness of their unlabeled DNA to labeled DNA from the type strains of *C. jejuni*, *C. coli*,

TABLE 2. Comparison of methods for hippurate hydrolysis on *Campylobacter* strains by using DNA relatedness to identify species

Strain no.	Species	Identification by:				% DNA relatedness (RBR) with type strains[a]:								
						C. jejuni D133			*C. coli* D145			*C. laridis* D146		
		Skirrow method	CDC method	Harvey method	GLC method	50°C	65°C	%D[b]	50°C	65°C	%D[b]	50°C	65°C	%D[b]
D109	*C. jejuni*	+	+	+	+	68	66	1.0	28	18	8.0	6		
D113	*C. jejuni*	+	+	+	+	82	75	1.5	37					
D114	*C. jejuni*	+	+	+	+	82	77	1.0	37	21	12.0	17		
D116	*C. jejuni*	+	+	+	+	90	90	0.5	32			17		
D117	*C. jejuni*	+	+	+	+		71		38					
D121	*C. jejuni*	+	+	+	+	71	90	1.5	36			17		
D123	*C. jejuni*	+	+	+	+	94	90	1.5	35			22		
D125	*C. jejuni*	+	+	+	+	84	84	0.5	26		9.5			
D127	*C. jejuni*	v (50)[c]	v (17)	−	+w	84	81	0.5	32	17		18		
D128	*C. jejuni*	v (50)	v (50)	−	+w	86	82	1.5	58					
D129	*C. jejuni*	+	+	+	+	92	95	1.0	37			12		
D131	*C. jejuni*	+	+	+	+	76	72	1.0	54					
D132	*C. jejuni*	+	+	+	+	72	72	1.0	35					
D133	(CIP 702[T])[d]	+	+	+	+	100	100	0.0	48	36	12.0			
D135	*C. jejuni*	+	+	+	+	75	76	2.0	39	24	8.5			
D136	*C. jejuni*	+	+	+	+	90	85	1.5		25	10.0			
D139	*C. jejuni*	+	+	+	+	72	76	0.5	35	27	10.5			
D140	*C. jejuni*	+	+	+	+	72	76	0.5	38	24				
D141	*C. jejuni*	+	+	+	+	82	81	1.5	56		12.0			
D142	*C. jejuni*	−	v (86)	+	+	79	81	1.5	58	47	10.0			
D143	*C. jejuni*	+	+	+	+	85	87	1.5	37					
D144	*C. jejuni*	+	+	+	+	93	73	1.0	36			21		
D111	*C. coli*	−	−	−	−		38		78	70				
D112	*C. coli*	−	−	−	−	48	36	9.5	83	73	1.0			
D115	*C. coli*	−	−	−	−		37		86	87	1.5			
D118	*C. coli*	−	−	−	−	48	27	11.5	60	60	6.5			
D122	*C. coli*	−	−	−	−		34		78	78	0.0			
D124	*C. coli*	−	−	−	−	75	63	7.0	89	81	1.0			
D126	*C. coli*	−	−	−	−	41	27	10.5	76	75	0.0			
D130	*C. coli*	−	−	−	−	53	33	11.0	62	60	6.0			
D134	*C. coli*	−	−	−	−	49		11.0	93		2.5			
D137	*C. coli*	−	−	−	−	48	31	11.0	77	78	0.5			
D145	(CIP 7080[T])[d]	−	−	−	−	50	30	9.5	100	100	0.0	16		
D146	(NCTC11352[T])[d]	−	−	−	−	16	6	15.0	16		7.0	100	100	0.0
D67	*C. laridis*	−	−	−	−							82	81	1.5
D70	*C. laridis*	−	−	−	−							82	92	2.0
D71	*C. laridis*	−	−	−	−							95	84	2.5
SK987	*C. laridis*	−	−	−	−				19			56	73	ND[e]
SK354	*C. laridis*	−	−	−	−							79	81	2.5
F2895	*C. laridis*	−	−	−	−	32						73	86	3.5
D110	*C. laridis*	−	−	−	−	26		13.5	22			73	90	0.5

[a] RBR, Relative binding ratio: (% heterologous DNA bound to hydroxyapatite/% homologous DNA bound to hydroxyapatite) × 100.
[b] D, Decrease in thermal stability of heterologous DNA duplexes compared with that of homologous duplex on the assumption that ech 1°C decrease in stability is caused by approximately 1% of unpaired bases (13). D is calculated to the nearest 0.5%. All tests were done at least twice.
[c] v, Variable; values in parentheses are percent positive of times tested. The times positive/times tested were strain D127, 1/2 (Skirrow) and 2/12 (CDC); strain D128, 1/2 (Skirrow) and 4/8 (CDC); and strain D142, 6/7 (CDC). +, Positive; +w, weakly positive; −, negative.
[d] T, Type strain.
[e] ND, Not done.

and *C. laridis* (Table 2). Six of seven *C. laridis* strains were 70% or more related to the *C. laridis* type strain in the reactions at 50 and 65°C; the other strain was 56% related at 50°C and 73% related at 65°C. The divergence in related DNA sequences was 0.5 to 3.5%. Eight of ten strains identified as *C. coli* were 75% or more related to the *C.coli* type strain at both 50 and 65°C. Similarly, 21 strains identified as *C. jejuni* showed substantially more relatedness to the type strain of *C. jejuni* (68 to 94% at 50°C and 66 to 85% at 65°C) than to that of *C. coli* (26 to 58% at 50°C and 17 to 47% at 65°C). The divergence in related sequences was 0.0 to 2.0% in reactions with *C. jejuni* and 8.0 to 12.0% in reactions with *C. coli*.

Hippurate hydrolysis results by all four methods are shown in Table 2 and summarized in Table 3. The 11 *C. coli* and 7 *C. laridis* strains were negative by each of the three tube methods and by the GLC method. Results for the 22 *C. jejuni* strains were not as clearly defined. Although all *C. jejuni* isolates were positive by at least one method (the GLC method), two isolates were weakly positive by this method each time they were tested (strains D127 and D128). Three strains, D127, D128, and D142, gave different results in the

TABLE 3. Hippurate hydrolysis results for 41 thermophilic *Campylobacter* spp. by four methods

Species identification by DNA relatedness	No. of strains tested	Reaction[a]	No. of strains by: Skirrow method	CDC method	Harvey method	GLC method
C. jejuni	22	+	19	19	20	22
		−	1	0	2	0
		v	2	3	0	0
C. coli	11	−	11	11	11	11
C. laridis	8	−	8	8	8	8

[a] +, Positive reaction; −, negative reaction; v, variable reaction.

tube procedures; however, two of these strains were weakly positive by the GLC method.

Twenty strains selected for reproducibility studies showed reproducible results, except for three strains noted in Table 2. These three strains were tested additional times (Table 2).

The pH of the substrate solution has been reported to influence results in the hippurate test (11; T. Smeltzer, personal communication); therefore, selected strains were tested by the CDC method with sodium hippurate dissolved in phosphate-buffered saline (pH 6.4 and 6.8) and compared with the CDC method involving the use of hippurate dissolved in water (pH 5.3). Cultures were tested after 24 and 48 h of incubation (Table 4). No differences were noted with the various diluents when a 24-h culture was used. However, the diluent influenced the results for strain D142 when a 48-h culture was used. Buffered diluent was inconsistent with results obtained with distilled water and the identity of the organism (Table 4).

Strains used in this study were phenotypically characterized by 22 tests (Table 5). Two *C. laridis* strains gave aberrant reactions on phenotypic tests. One strain, SK987, was susceptible to cephalothin, and a second strain, SK354, was catalase negative and susceptible to nalidixic acid. Both strains were compatible with *C. laridis* in their DNA relatedness.

DISCUSSION

It is difficult to differentiate among these three species by phenotypic characteristics. Although the *C. coli* strains were more likely to grow aerobically than *C. jejuni* and *C. laridis* strains, this is a poor test because laboratory strains become more tolerant of air after repeated subcultures. Hippurate hydrolysis and nalidixic acid susceptibility are the two most important phenotypic characteristics. One of eight *C. laridis* strains was susceptible to nalidixic acid; therefore, this test is not completely reliable for differentiation purposes. For this reason it was necessary to resort to DNA hybridization to definitively separate the three species. The results for three of the strains deserve comment. Strain D124 was substantially related to *C. jejuni* and to *C. coli* (75% versus 89% at 50°C; 63 versus 81% at 65°C). Divergence was 7% with *C. jejuni* and only 1.0% with *C. coli*. Therefore, it appears that this strain is *C. coli* or *C. coli*-like. Two other strains, D118 and D130, were about 60% related to *C. coli* in reactions at 50°C, and although relatedness remained 60% at 65°C, divergence was 6.0 to 6.5%. These strains were about 50% related to *C. jejuni* at 50°C and about 30% at 65°C, and showed 11.0 to 11.5% divergence. Similar relatedness values were obtained when reactions on each of these strains were done three times on both the original and a second DNA preparation. They may be genetically atypical *C. coli* or a species closely related to, but distinct from, *C. coli*, but they are clearly not *C. jejuni*. All three of these atypical *C. coli* strains were hippurate negative. It is clear that further study of the relatedness of these strains is necessary.

Hippurate hydrolysis is the most useful test for separating *C. jejuni* from *C. coli* and *C. laridis*. The problem is with test-to-test variation with a minority of strains when tested by the tube method. The GLC method for hippurate hydrolysis is sensitive for detecting small amounts of benzoic acid, and results of this method corresponded better to the identity of *Campylobacter* sp. by DNA relatedness studies than did results of the three tube methods. It is important that no false-positives were seen by any of the three tube methods; however, at least two false-negatives were seen with each tube procedure. On the basis of these findings, the sensitivities of the three tube methods are comparable. Either of the rapid-tube methods (Harvey and CDC) was preferred to the Skirrow method, which required more time for preparation

TABLE 4. Comparison of reactions in different hippurate diluents

Strain no.	Identification by DNA relatedness	Reaction[a] with buffered hippurate at: pH 6.4 24 h	pH 6.4 48 h	pH 6.8 24 h	pH 6.8 48 h	Reaction[a] with hippurate in water (pH 5.3) 24 h	48 h
D127	*C. jejuni*	−	−	−	−	−	−
D128	*C. jejuni*	+	+	+	+	+	+
D142	*C. jejuni*	+	−	+	−	+	+
D133	*C. jejuni*	+	+	+	+	+	+
D145	*C. coli*	−	−	−	−	−	−

[a] +, Positive reaction, −, negative reaction.

TABLE 5. Phenotypic characteristics of strains

Characteristic	% Positive for: *C. jejuni* (*n* = 22)	*C. coli* (*n* = 11)	*C. laridis* (*n* = 8)
Aerobic growth	18[a]	73[a]	0
Microaerobic growth (5% O_2)	100	100	100
Anaerobic growth	5	0	0
Growth on MacConkey agar	100	100[b]	100
Oxidase	100	100	100
Catalase	100	100	88
Motile	77	100	88
Visible stained flagella	77	100	88
Nitrate reduction	100	100	100
H_2S, TSI	0	9[c]	0
H_2S, lead acetate	100	100	100
Growth in 1% glycine	100	100	100
Growth in 3.5% NaCl	0	0	0
Growth at 25°C	0	0	0
Growth at 36°C	100	100	100
Growth at 42°C	100	100	100
Fermentation of glucose	0	0	0
Oxidative utilization of glucose	0	0	0
Gram-negative spiral rod	100	100	100
Nalidixic acid, sensitive	100	100	12
Cephalothin, sensitive	0	0	12
Hippurate hydrolysis	100	0	0

[a] Light growth, usually 1+ on a scale of 1+ to 4+.
[b] Trace amount of growth on 2 of the 11 strains.
[c] Trace amount of H_2S.

of the inoculum and performance of the test. Previous investigators have reported the isolation of hippurate-negative *C. jejuni* (5, 6, 12). One of the strains in this study (D127) is a subculture of strain PC95 found to be hippurate negative by Hébert et al. (6); therefore, it is possible that in some cases previous findings of hippurate-negative *C. jejuni* strains were due to use of insufficiently sensitive tests. The tube tests are satisfactory for routine use; however, the GLC method is recommended when questionable results are obtained with the tube test.

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