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Evaluation of the Indoxyl Acetate Hydrolysis Test for Rapid Differentiation of *Campylobacter*, *Helicobacter*, and *Wolinella* Species

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A total of 410 well-defined *Campylobacter*, *Helicobacter*, and *Wolinella* strains, comprising 26 named species, subspecies, and defined groups, were tested for indoxyl acetate hydrolysis by a disk method by using disks prepared at the Centers for Disease Control, Atlanta, Ga. All *C. coli* (43 strains), *C. cryaerophila* (34 strains), *C. fennelliae* (5 strains), *C. fennelliae–Campylobacter*-like organism 3 (2 strains), *C. jejuni* (66 strains), *C. jejuni* subsp. *doylei* (3 strains), hippurate-negative *C. jejuni-C. coli* (15 strains), "*C. upsaliensis*" (39 strains), *H. mustelae* (5 strains), *W. curva* (1 strain), and *W. recta* (1 strain) hydrolyzed indoxyl acetate. Four strains gave weak positive reactions, and the remaining 196 strains, which belonged to 15 species, subspecies, and defined groups, gave negative reactions. Of the 410 study strains, 246 and 125 strains were tested for indoxyl acetate hydrolysis by a disk method and a tube method, respectively, by using commercially produced disks. The disk method, regardless of source, required less time and interpretation than the tube method did. Better differentiation between *Campylobacter* spp. was obtained with the indoxyl acetate test than with the trimethylamine *N*-oxide test. The indoxyl acetate disk distinguished *C. lari* from *C. jejuni* and *C. coli*, *C. cinaedi* from *C. fennelliae*, and *H. pylori* from *H. mustelae* and suggested that *W. succinogenes* could be differentiated from *W. recta* and *W. curva*. The indoxyl acetate disk method could be performed in 5 to 30 min, was easy to read and interpret, and should be useful as a routine diagnostic test for identification of *Campylobacter* spp.

The increasing awareness of the importance of *Campylobacter* spp. in human and animal infections has prompted workers in many laboratories to develop tests for the rapid and reliable identification of *Campylobacter* spp. and members of related genera. Their identification by conventional biochemical methods is difficult because they are relatively biochemically inert.

Indoxyl is a product of the putrefactive decomposition of tryptophan in the intestines of humans through bacterial action (21). Several useful diagnostic tests based on the hydrolysis of indoxyl compounds have been developed recently: indoxyl sulfate for identification of *Providencia stuartii* and *Klebsiella pneumoniae* (7); indoxyl butyrate for identification of *Moraxella catarrhalis* (6); and indoxyl-β-D-glucuronide for identification of *Escherichia coli* (8, 13, 39). Bacterial hydrolases release indoxyl from these compounds. In the presence of air (oxygen), indoxyl forms into indigo white and indigo (7), and the appearance of a dark blue color indicates that the indoxyl compound was metabolized.

In 1987, Mills and Gherna (16) developed a rapid indoxyl acetate hydrolysis test to distinguish the *Campylobacter* spp. They examined 112 strains from 12 different species. However, their study did not include several newly recognized or proposed *Campylobacter* spp., including "*C. upsaliensis*," *C. jejuni* subsp. *doylei*, *Helicobacter mustelae* (formerly *Campylobacter mustelae*), *Campylobacter*-like organisms (CLOs), or the closely related *Wolinella* spp.

No data are available on indoxyl acetate hydrolysis by all currently described *Campylobacter* spp. and members of closely related genera tested under the same laboratory conditions. The purpose of this study was to evaluate the indoxyl acetate test by using a large number of well-defined strains and to establish its applicability and importance in the rapid differentiation of *Campylobacter* and related genera.

MATERIALS AND METHODS

Strains. A total of 410 well-defined *Campylobacter*, *Helicobacter*, and *Wolinella* strains, comprising 26 named species, subspecies, and defined groups, including 23 type or reference strains, were tested. All strains were obtained from the collection of the Campylobacter Reference Laboratory, Centers for Disease Control (CDC), Atlanta, Ga. The sources and numbers of strains included in the study are indicated in Table 1. In this report *C. cryaerophila* includes *C. cryaerophila* and aerotolerant CLOs. "*C. upsaliensis*" and "*C. sputorum* subsp. *faecalis*" are indicated as proposed species by their enclosure in quotation marks.

Maintenance and growth of strains. Frozen stock cultures were maintained in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) with 20% glycerol at −70°C. All strains were subcultured onto heart infusion agar (HIA) with 5% defibrinated rabbit blood (HIA-RB; BBL) and incubated in an atmosphere of approximately 5% O_2, 7.5% CO_2, 7.5% H_2, and 80% N_2. All strains were incubated at 36°C, except for *C. cryaerophila* and *C. nitrofigilis*, which were cultured at 30 and 25°C, respectively. All strains showed adequate growth after 24 or 48 h of incubation, except for *H. pylori* and *Wolinella* spp., which required 72 to 120 h incubation.

Test procedures. Strains were tested by (i) a disk method by using disks prepared at CDC (method 1), (ii) a disk method by using disks produced commercially (American Type Culture Collection [ATCC]), Rockville, Md. (method 2), and (iii) a tube method by using the disks from ATCC (method 3). The disks were prepared at CDC by using a 10%

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TABLE 1. Source and number of *Campylobacter*, *Helicobacter*, and *Wolinellla* strains examined for indoxyl acetate hydrolysis

Organism	No. of strains[a]									
	Tested	Human clinical source				Animal				Environmental	Unknown
		B	F	M	U	P	Bo	Po	O		
C. cinaedi	13	6	6	1							
C. concisus	1			1							
C. coli	43	4	20	2	5	6	1	4			1
C. cryaerophila[b]	34	2	13	3		5	3		8		
C. fennelliae	5		5								
C. fennelliae-CLO 3	2	1	1								
C. fetus subsp. *fetus*	35	21	3	10					1		
C. fetus subsp. *venerealis*	5						4				1
C. hyointestinalis	64	5	17			39	3				
C. jejuni	66	1	21		23		4	8	7		2
C. jejuni subsp. *doylei*	3	1	2								
C. jejuni-C. coli, hippurate negative	15	2	12								1
C. lari	35	4	15		3			1	4	8	
C. mucosalis	4					3			1		
C. nitrofigilis	1									1	
C. sputorum subsp. *bubulus*	7						6				1
C. sputorum subsp. *sputorum*	3			2							1
"*C. sputorum* subsp. *fecalis*"	5								5		
"*C. upsaliensis*"	39	22	16						1		
CLO	2		1					1			
CLO 1B	3	1	2								
H. mustelae	5								5		
H. pylori	17			17							
W. curva	1			1							
W. recta	1			1							
W. succinogenes	1						1				

[a] Abbreviations: B, blood; F, feces; M, miscellaneous; U, unknown; P, pig, Bo, bovine; Po, poultry; O, other.
[b] *C. cryaerophila* and aerotolerant CLOs.

(wt/vol) solution of indoxyl acetate (Sigma Chemical Co., St. Louis, Mo.) in acetone; 50 μl of the solution was added to each blank paper disk (diameter, 0.6 cm; BBL). After drying at room temperature, the disks were stored at 4°C in a brown tube with a desiccant.

The test procedures for the disk method were the same regardless of the source of the disks. A large loopful of growth from an HIA-RB plate was placed onto the disk, and a drop of sterile distilled water was added. Hydrolysis of indoxyl acetate was indicated by the development of a dark blue color in 5 to 10 min. Weak positive reactions were characterized by the appearance of a pale blue color in 10 to 30 min. No color change indicated no hydrolysis and a negative result.

To perform the tube procedure, a large loopful of growth from the HIA-RB plate was emulsified in 0.3 ml of sterile distilled water, and the ATCC disk was added to the suspension. Test results were read in the same way as described above for the disk method, except that positive and weak reactions required 10 to 45 min.

All strains (n = 410) were tested by method 1, 246 strains were tested by method 2, and 125 strains were tested by method 3. The *C. jejuni* type strain ATCC 33560 and the *C. lari* type strain ATCC 35221 (formerly *C. laridis* [37]) were included as positive and negative controls for each group of strains tested by all three methods.

RESULTS

Of the strains that we analyzed, 311 (75%) were from 40 states in the United States and 80 (20%) were from 13 other countries. We were not able to trace the geographic sources of the remaining 19 strains (5%). Of the 273 human strains, 134 (49%) were isolated from feces or rectal swabs, 70 (26%) strains were from blood, 38 (14%) strains were from miscellaneous sites within the human body (peritoneal fluid, joint fluid, groin abscess, or cerebrospinal fluid), and 31 (11%) were from unknown clinical sources. There were 121 isolates from animals; most were from porcine and bovine sources. There were nine environmental strains (Table 1).

Results of indoxyl acetate hydrolysis for 410 *Campylobacter*, *Helicobacter*, and *Wolinella* strains by using disks prepared at CDC are indicated in Table 2. A uniform reaction was recorded by all strains within one species with the exception of two *C. jejuni-C. coli* (hippurate negative) and two *C. fennelliae*-CLO 3 strains. These four strains gave weak positive reactions, while the remaining 13 *C. jejuni-C. coli* (hippurate negative) strains gave a positive reaction. No other *C. fennelliae*-CLO 3 strains were tested. All *C. coli* (43 strains), *C. cryaerophila* (34 strains), *C. fennelliae* (5 strains), *C. jejuni* (66 strains), *C. jejuni* subsp. *doylei* (3 strains), "*C. upsaliensis*" (39 strains), *H. mustelae* (5 strains), *W. curva* (1 strain), and *W. recta* (1 strain) hydrolyzed indoxyl acetate. The remaining 196 strains, belonging to 15 species, subspecies, and defined groups, were negative for indoxyl acetate hydrolysis.

The CDC disk results were compared with those obtained with the commercially available indoxyl acetate disks by using 246 strains representing 19 species, subspecies, and defined groups. The results are summarized in Table 2. The

TABLE 2. Indoxyl acetate hydrolysis by *Campylobacter*, *Helicobacter*, and *Wolinella* spp. by disk and tube methods by using CDC and ATCC disks

Organism	No. of strains[a]											
	DNA	Indoxyl acetate test response										
		D-CDC				D-ATCC				T-ATCC		
		Tested	+	−	W	Tested	+	−	W	Tested	+	−	W
C. coli	25	43	43			19	19			10	10		
C. cryaerophila[b]	32	34	34			20	20			10	10		
C. fennelliae	2	5	5										
C. fennelliae-CLO 3		2			2	2			2	2			2
C. jejuni	37	66	66			31	31			24	24		
C. jejuni subsp. *doylei*	1	3	3			3	3			3	3		
C. jejuni-C. coli, hippurate negative		15	13		2	15	13		2	5	3		2
"*C. upsaliensis*"	13	39	39			28	28			28	28		
H. mustelae		5	5			5	5			5	5		
W. curva		1	1			1	1			1	1		
W. recta		1	1										
C. cinaedi	5	13		13		8		8		8		8	
C. concisus		1		1		1		1		1		1	
C. fetus subsp. *fetus*	15	35		35		20		20		10		10	
C. fetus subsp. *venerealis*		5		5									
C. hyointestinalis	5	64		64		47		47		4		4	
C. lari	9	35		35		19		19		6		6	
C. mucosalis		4		4		2		2		2		2	
C. nitrofigilis		1		1		1		1		1		1	
C. sputorum subsp. *bubulus*		7		7		5		5		4		4	
C. sputorum subsp. *sputorum*		3		3									
"*C. sputorum* subsp. *fecalis*"		5		5		3		3					
CLO		2		2									
CLO 1B		3		3									
H. pylori		17		17		14		14					
W. succinogenes		1		1		1		1		1		1	

[a] Abbreviations and explanations: DNA, strains identified by DNA homology; D-CDC, disks prepared at Centers for Disease Control; D-ATCC, disks commercially available from ATCC; T-ATCC, tube method with ATCC disks; +, positive reaction; −, negative reaction; w, weak positive reaction.
[b] *C. cryaerophila* and aerotolerant CLOs.

source of the indoxyl acetate disks that we used did not alter the outcomes of the tests. All strains consistently showed the same positive, weak positive, or negative reactions with the ATCC and CDC disks.

We also compared the tube method with the disk procedures using a total of 125 strains comprising 18 species, subspecies, and defined groups. We did not observe any significant discrepancies in the results obtained by this method when they were compared with those obtained by the disk procedures (Table 2). However, among 84 stains which gave positive reactions by the tube method, the color change occurred within 15 min in only six strains. The remaining 78 strains required 15 to 45 min for development of the color indicative of indoxyl acetate hydrolysis.

DISCUSSION

In recent years, we have witnessed an increased interest in *Campylobacter* spp. and members of the related genera *Helicobacter* and *Wolinella*. Some of the species are well-established human pathogens (24), and with the continued addition of new species to these genera, it is important to develop more rapid, sensitive, and specific tests to isolate and identify those species and subspecies that are generally accepted as important human pathogens.

The strains analyzed in this study were from 13 countries and 40 states in the United States. The fact that bacterial strains within one species or group but from diverse geographical and biological sources responded with uniform and consistent results suggests that the indoxyl acetate test is a reliable differential method. Our results correlate with those of Mills and Gherna (16), except that we did not observe weakly positive *C. cinaedi* strains, whereas one of four *C. cinaedi* strains was weakly positive in their study.

C. jejuni and *C. coli* are well-established etiologic agents associated with gastroenteritis in humans (3, 4). *C. lari*, another member of the thermophilic campylobacters (2), has been increasingly isolated from human clinical specimens and may be an important pathogen for both normal and immunosuppressed human hosts (18, 26, 31). Hippurate hydrolysis and nalidixic acid susceptibility are considered the two most important phenotypic characteristics for differentiating strains of *C. jejuni*, *C. coli*, and *C. lari*. *C. lari* strains are nalidixic acid resistant, and *C. jejuni* and *C. coli* strains are nalidixic acid susceptible (27). However, nalidixic acid-susceptible *C. lari* and nalidixic acid-resistant *C. jejuni* and *C. coli* strains have also been described (14, 15, 17, 35, 38). Our study included 10 nalidixic acid-resistant *C. jejuni* strains. These 10 *C. jejuni* strains, like all *C. jejuni* and *C. coli* strains in this study, gave positive results in the indoxyl acetate test, while all the *C. lari* strains were indoxyl acetate negative. These results imply that the indoxyl acetate test is more reliable than nalidixic acid susceptibility for differentiating the thermophilic *Campylobacter* species.

Indoxyl acetate hydrolysis was a constant feature of *C. jejuni*, *C. coli*, and *C. jejuni-C. coli* strains, regardless of their ability to hydrolyze sodium hippurate. Included in this

study were 9 hippurate-negative *C. jejuni* strains previously identified by DNA homology studies (34) and 15 hippurate-negative *C. jejuni-C. coli* strains. All 24 strains hydrolyzed indoxyl acetate, with 2 strains consistently giving weak positive reactions. Therefore, the indoxyl acetate test does not enable one to distinguish hippurate-negative *C. jejuni* from *C. coli* strains; genetic methods remain the only reliable means for separating these species.

With the development of selective media and culture techniques for fecal specimens, several new *Campylobacter* spp. have been identified and associated with human disease. Among these newly described pathogens are *C. cinaedi* and *C. fennelliae* (5, 20, 33). However, there are few dependable phenotypic characteristics to differentiate these two species. Currently, the biochemical feature most frequently used to distinguish them is the nitrate reduction test, with *C. cinaedi* strains being nitrate reducers (positive result) and *C. fennelliae* strains being incapable of reducing nitrate (negative result). In our study, none of the *C. cinaedi* strains hydrolyzed indoxyl acetate, while all strains of *C. fennelliae* and *C. fennelliae*-CLO 3 strains were indoxyl acetate positive. This test is an additional tool that is useful in the rapid differentiation of these two species.

C. cryaerophila, formerly a group of aerotolerant CLOs associated with animal abortions, was designated a new species in 1985, and subsequently, a single case of human diarrhea has been associated with this species (19, 32). Biochemically, *C. cryaerophila* resembles *C. fetus* subsp. *fetus* in that both species grow at 25°C and neither species grows well at 42°C. All 34 strains of *C. cryaerophila* were indoxyl acetate positive, and all *C. fetus* subsp. *fetus* (35 strains) were indoxyl acetate negative in this study. Therefore, the indoxyl acetate test is an additional means of distinguishing these two species.

Members of the genus *Wolinella* are strongly associated with human adult periodontitis, with *W. recta* and *W. curva* having been isolated from periodontal pockets, oral cavities, and cultures of blood samples from patients with progressive periodontal disease (12, 30). *C. concisus* shares many characteristics with members of the genus *Wolinella* (28). It is often found in human gingival crevices (22) and has been identified in at least one nonoral specimen from a human, a foot ulcer (11). Fecal carriage of *C. concisus* is common and may be associated with gastrointestinal disorders (36). In a study conducted by Tanner et al. (29), *C. concisus* and *W. recta* could be distinguished more reliably on the basis of sodium dodecyl sulfate-polyacrylamide gel electrophoresis than it could by biochemical tests. We emphasize that even though our study included only one strain each of *W. recta*, *W. curva*, and *C. concisus*, the results suggest that indoxyl acetate hydrolysis could facilitate differentiation of *C. concisus* from *W. recta* and *W. curva*. *C. concisus* gave a negative reaction and both *W. curva* and *W. recta* gave positive reactions in this test. *W. succinogenes* has been isolated only from the bovine rumen (28), and *W. curva* is found in humans (30). These two species have similar biochemical characteristics. In our study the *W. succinogenes* strain was indoxyl acetate negative, and the *W. curva* strain was indoxyl acetate positive. The indoxyl acetate test, along with knowledge of the origin of the strain, can be useful in rapidly differentiating *W. succinogenes* from *W. curva*.

H. pylori is strongly associated with type B gastritis and peptic ulcer disease in humans throughout the world (23). Isolates obtained from the stomachs of ferrets in the United States and Australia have been classified as *H. mustelae* (9, 10). There are only a few biochemical features to distinguish these two species. They differ in their ability to grow anaerobically and at 42°C and in their reduction of nitrites and exhibition of leucine arylamidase. In this study, we distinguished these species by the indoxyl acetate hydrolysis test; the 17 *H. pylori* strains were negative, and the 5 *H. mustelae* strains were positive.

In 1983, Benjamin et al. (2) described a test for anaerobic growth of *Campylobacter* spp. in the presence of trimethylamine *N*-oxide hydrochloride (TMAO). At that time, *C. lari* was the only reported *Campylobacter* spp. capable of utilizing the oxygen in TMAO medium and thereby attaining growth in an anaerobic atmosphere. Subsequently, other species have been shown to be positive by the TMAO test (25), including the species *C. fetus* subsp. *fetus*, *C. hyointestinalis*, *C. sputorum* (all three subspecies), and *C. mucosalis* reported from our laboratory (1). Since that report was published, we tested an additional 185 *Campylobacter* species, subspecies, and CLOs and 7 additional *H. pylori* strains. We found one *C. lari* strain to be TMAO negative and strains of five other species (*C. jejuni*, *C. jejuni* subsp. *doylei*, *C. coli*, *C. fetus* subsp. *venerealis*, and *C. cryaerophila*) to be TMAO positive.

Our results indicate that the indoxyl acetate test is a more useful and reliable differential test for *Campylobacter* and related genera than the TMAO test is. The indoxyl acetate test distinguished *C. lari* from *C. jejuni* and *C. coli*, *C. cinaedi* from *C. fennelliae*, and *H. pylori* from *H. mustelae* and suggested that *W. succinogenes* (one strain) could be differentiated from *W. recta* and *W. curva* (one strain each). The indoxyl acetate test can be performed in 5 to 30 min, as compared with the 7 days required for the TMAO test, and is easier to read and interpret. Therefore, we have replaced the TMAO test with the indoxyl acetate test in our routine battery of diagnostic tests for *Campylobacter* identification.

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