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Assessment of Enzyme Detection Tests Useful in Identification of Campylobacteria

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Twenty-one type or other reference strains, each representing a different *Campylobacter*, *Helicobacter*, or *Arcobacter* taxon, and a reference strain of *Staphylococcus aureus* were used to assess the reproducibility of nine enzyme detection tests used in the identification of campylobacters. For five of the tests (alkaline phosphatase, DNase, and H_2S production, indoxyl acetate hydrolysis, and nitrate reduction), more than one procedure was employed to determine the most suitable method. Alkaline phosphatase test results were better defined and more reproducible if read after 1 h of incubation. Detection of DNase was fully reproducible with each method (except with *Helicobacter pylori*), but reactions were generally weaker than those of other DNase-producing organisms. Both procedures for determining H_2S production were irreproducible for the same strains. The reproducibility of indoxyl acetate hydrolysis was improved by using disks impregnated with 25 μl of substrate. Reduction of nitrate was best determined by Cook's plate method. Results for the other tests examined (catalase, oxidase, and urease production and hippurate hydrolysis) were both pertinent and fully reproducible for all strains.

The taxonomy of the genus *Campylobacter* has recently undergone considerable revision, and at present, 11 species, including two former *Wolinella* species, may be considered members of this genus (17). Certain organisms formerly classified as *Campylobacter* species have been proposed as constituting two different genera, *Helicobacter* and *Arcobacter* (17). In addition, the family *Campylobacteraceae* has been proposed to include the genera *Campylobacter* and *Arcobacter* and to exclude the genus *Helicobacter* (16), although all of these groups may be referred to as "campylobacteria" or "*Campylobacter*-like organisms."

Identification of these organisms in clinical laboratories may prove difficult, since strains of these bacteria have relatively fastidious growth requirements and are asaccharolytic. Most of the discriminatory phenotypic tests, e.g., tests of growth requirements, resistance to various chemicals (including antibiotics), and temperature tolerances, rely on growth inhibition; identification of a strain may therefore require several days. Only a limited number of enzyme detection tests have been found to discriminate adequately between these organisms, although several are included in most campylobacter identification schemes. Differentiation of *Campylobacter coli* from *C. jejuni* subsp. *jejuni*, for example, relies almost entirely on hippurate hydrolysis.

The importance of reproducibility in such key tests is self-evident; however, in the absence of standard methods, most workers employ a procedure unique to their laboratory. Differences in methods used may lead to a different outcome for nominally the same test. This may explain discrepancies seen in phenotypic data published for campylobacters. As an example, the percentage of strains of *C. coli* reported to hydrolyze DNA ranges from 16% (1) to 75% (14). Similarly, *Helicobacter pylori* (formerly *C. pylori*) was originally described as urease negative (9), although it is now accepted that this enzyme is present in a copious and potent form in this species.

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We have previously reported the influence of both inoculum size and basal medium on certain phenotypic characters of campylobacters, primarily in growth inhibition tests (11, 12). The aim of the present study was to establish reproducible methods for enzyme detection tests employed in the identification of such organisms.

The 22 strains used in this study and their sources are listed in Table 1 (16 are type strains). All were grown for 3 days in anaerobic jars in which a microaerophilic atmosphere was created by reducing the atmospheric pressure to 540 mm Hg (1 mm Hg = 133.322 Pa) and regassing with an anaerobic gas mixture (80% nitrogen, 10% hydrogen, 10% carbon dioxide) and incubated at 37°C, except for *Arcobacter nitrofigilis* CCUG 15893, which was incubated at 25°C, the optimum growth temperature for this species. The culture medium used was nutrient broth no. 2 (Oxoid Ltd., Basingstoke, England) with 5% (all percentages are expressed as vol/vol or wt/vol where applicable) whole horse blood added after the autoclaved medium was cooled to approximately 50°C; 2% Japanese agar was used as the gelling agent.

Alkaline phosphatase was detected by the method of Itoh et al. (7); results were read at 1, 2, 3, and 24 h, and any yellow coloration was regarded as a positive result. Catalase production was tested by placing a small amount of bacteria on a plastic or glass rod into a drop of 6% hydrogen peroxide within a petri dish; a positive reaction was defined as an immediate appearance of copious bubbles. Weak positive (slow or immediate appearance of few bubbles) and negative results were also recorded. All strains were tested for DNase activity by the methods of Holmes et al. (6) (method i) and Lior and Patel (8) (method ii). In addition, strains NCTC 6571, NCTC 11351, NCTC 11415, NCTC 11637, and NCTC 12145 were also examined by the method of Hänninen (5) (method iii) and a method using unmodified DNase agar (Difco Laboratories Ltd., East Molesey, England) heavily inoculated with bacteria over a 1.5-cm^2 area and developed with 1 N HCl (BDH Ltd., Poole, England) (method iv). The

TABLE 1. Strains used in this study

Organism	Strain designation (equivalent[s])[a]	Source
A. cryaerophila	NCTC 11885^T (CCUG 12012)	Aborted bovine fetus
A. nitrofigilis	NCTC 12251^T (CCUG 15893)	Root of salt marsh plant
C. coli	NCTC 11366^T (CIP 7080)	Pig feces
C. concisus	NCTC 11485^T (ATCC 33237)	Human gingival sulcus
C. fetus subsp. *fetus*	NCTC 10842^T (CIP 5396, ATCC 27374)	Sheep fetus brain
C. fetus subsp. *venerealis*	NCTC 10354^T (ATCC 19438)	Heifer vaginal mucus
C. hyointestinalis	NCTC 11608^T	Pig intestine
C. jejuni subsp. *doylei*	NCTC 11951^T (IMVS 1141)	Feces from a 2-yr-old child
C. jejuni subsp. *jejuni*	NCTC 11351^T (CIP 702)	Bovine feces
C. jejuni subsp. *jejuni*	NCTC 12145	Unknown
C. lari	NCTC 11352^T (ATCC 35221)	Cloacal swab from a herring gull
C. mucosalis	NCTC 11000^T	Pig small intestine
C. sputorum bv. bubulus	NCTC 11367 (CIP 53103)	Bull sperm
C. sputorum bv. fecalis	NCTC 11415	Sheep feces
C. sputorum bv. sputorum	NCTC 11528^T (ATCC 35980, CCUG 9728)	Human oral cavity
C. upsaliensis	NCTC 11540	Dog feces
Campylobacter UPTC group[b]	NCTC 11845	River water
H. cinaedi	NCTC 11611	Rectal swab from a homosexual male
H. fenneliae	NCTC 11612^T (ATCC 35684)	Rectal swab from a homosexual male
H. mustelae	NCTC 12198^T	Ferret stomach
H. pylori	NCTC 11637^T	Human gastric antrum
S. aureus	NCTC 6571	Unknown

[a] ATCC, American Type Culture Collection, Rockville, Md.; CIP, Collection de l'Institut Pasteur, Paris, France; CCUG, Culture Collection of the University of Göteborg, Göteborg, Sweden; IMVS, Institute of Medical and Veterinary Science, Adelaide, Australia; NCTC, National Collection of Type Cultures, Central Public Health Laboratory, London, United Kingdom.

[b] UPTC, urease-positive thermophilic campylobacters.

Difco medium used in method iv served as the base for methods i to iii but was adapted accordingly. All DNase plates were incubated for 3 days. The presence of hippuricase was tested by the method of Skirrow and Benjamin (15) with the following modifications: bacterial cells were suspended in 0.8 ml of sterile distilled water; 0.2 ml of aqueous 5% sodium hippurate solution was added, the suspension was then incubated at 37°C for 4 h; and finally, 0.4 ml of 3.5% ninhydrin solution was added. A purple color was considered positive if it appeared within 10 min but was considered weakly positive if it appeared between 10 and 20 min. Hydrogen sulfide production in triple sugar iron medium (which examines for two enzymes, cysteine desulfhydrase and thiosulfate reductase) was tested by the method of Cowan (4), but the medium was inoculated with bacterial suspensions containing approximately 10^9 or 10^6 CFU/ml, prepared as previously described (11), and incubated at 37°C for 3 days. Blackening of at least 75% of the butt was regarded as positive, a trace of blackening (usually at the inoculum-slant interface only) was regarded as being weakly positive, and the absence of blackening was regarded as negative. Hydrolysis of indoxyl acetate was tested by method 1 of Mills and Gherna (10). In addition, a modification of this method was performed with disks impregnated with 25 μl of 10% indoxyl acetate solution. Reduction of nitrate was tested by three methods: (i) the method of Cook (3), employing an inoculum of approximately 10^6 CFU/ml and an incubation period of 24 h in a microaerophilic atmosphere; (ii) method 1 of Cowan (4), with the bacteria incubated in a microaerophilic atmosphere for 4 days; and (iii) the method of Itoh et al. (7). The presence of cytochrome oxidase was determined by the method of Bascomb et al. (2). Urease was detected by the rapid method of Owen et al. (13). All tests were performed three times on different occasions (freshly prepared plate media were used when appropriate) to assess reproducibility.

The percentage of aberrance (Table 2) for each test (except DNase) was calculated from the number of strains, of the 20 examined (NCTC 6571 and NCTC 12145 were used only as positive controls for the DNase test), which did not give a fully reproducible result (0 or 3 positives in 3 tests). The percentage of strains yielding reproducibly positive test outcomes was also calculated to indicate the discriminatory power of each test (Table 2). The percent correlation with data from the published type description (or other published sources in cases for which specific data are unavailable) is also provided in Table 2 as a guideline to assessing the results presented here. It should be emphasized that the type description data have been generated by using a wide variety of differing media and methods. The purpose of this study and our previous work (11, 12) was to determine and recommend methods that both were reproducible and offered maximum discrimination between the campylobacteria. Correlation of our results with previously published data was therefore not one of our aims. This should be considered when comparing our results with previously published data on these strains.

Alkaline phosphatase. At 1 h, only three strains were positive (NCTC 11612, NCTC 11637, and NCTC 12198), with an especially strong reaction in the last two strains. However, after 24 h all strains tested exhibited some yellow coloration, although some were more weakly colored than others. The percentage of aberrant results was highest after 2 h (25%) and lowest after 1 h (5%), largely because of the difficulty in establishing a clearly defined cutoff point. Certain strains gave only the faintest hint of color, and we suggest that such results should be regarded as false positives. Since the color developed with time, it seems prudent either to employ a spectrophotometric method to discriminate false-positive results from true positives or, alternatively, to read results earlier. We found that three strains (NCTC 11612, NCTC 11637, and NCTC 12198) consistently gave unequivocal positive results at 1 h of incubation, and

TABLE 2. Percentages of aberrance and reproducible positive results in eight enzyme detection tests for campylobacteria[a]

Test	Method (reference)	% Aberrance	% Reproducible positive results	% Correlation with published data
Alkaline phosphatase	1 h	5	15	90
	2 h	25	30	90
	3 h	25	35	75
	24 h	0	100	65
Catalase	See text	5	75	95
Hippuricase	Skirrow and Benjamin (15)	0	10	100
H_2S	10^9-CFU/ml inoculum	15	20	80
	10^6-CFU/ml inoculum	15	20	80
Indoxyl acetate hydrolysis	25 μl	10	25	90
	50 μl	20	25	80
Nitrate	Cook (3)	5	60	70
	Cowan method 1 (4)	25	55	65
	Itoh et al. (7)	0	100	85
Oxidase	Bascomb et al. (2)	0	100	100
Urease	Owen et al. (13)	0	20[b]	100

[a] Various methods were used for the tests. Correlations were made with data from published type descriptions or from other published sources when type descriptions were unavailable.
[b] Certain strains positive only after incubation for up to 6 h.

we recommend that this period be used when this test is performed without a spectrophotometric device.

Catalase, hippurate, oxidase, and urease. Although only one method was examined for each of these tests, the results were both as expected and highly reproducible (95 to 100%), which suggests that they have already been developed to their optimum; the only strain yielding an ambiguous result was NCTC 11000 (*C. mucosalis*) when examined for catalase. In addition, it was noted that an extended period of incubation was required (up to 6 h) for strain CCUG 15893 if urease was to be detected.

DNase. None of the strains examined (with the exception of those detailed below) exhibited DNase activity. This finding correlates with type descriptions. The results obtained for NCTC 11351, NCTC 12145, NCTC 11415, NCTC 11637, and NCTC 6571 are summarized in Table 3. Hydrolysis by all methods was reproducibly exhibited by NCTC 12145 (*C. jejuni* subsp. *jejuni*), which is recommended as a positive control strain for campylobacters (13a), and NCTC 6571 (*Staphylococcus aureus*). However, the degree of DNA hydrolysis was more intense with the latter strain. A faint pink hue was occasionally seen directly beneath the inoculum of NCTC 11637 with the methods described by Holmes et al. (6) and Lior and Patel (8). NCTC 11351, used as a negative control, was consistently negative, as was NCTC 11415. In addition, growth of strains NCTC 11351, NCTC 11415, and NCTC 11637 on all DNase-detecting media was either weak and irreproducible or absent. Unlike other workers (5, 8, 14), we found little evidence for DNase production in *C. sputorum* bv. fecalis and *H. pylori*. This finding may be linked to the ability of the strain to grow on the medium before the enzyme is produced. However, the Hänninen method (5) does not rely upon bacterial growth and furthermore has been used to demonstrate DNase activity of *H. pylori*. Our failure to reproduce the results obtained by these other workers suggests a serious shortcoming in the reproducibility of their test methods and furthermore suggests that this characteristic may not be one which can be attributed to either *C. sputorum* bv. fecalis or *H. pylori*. Since all test media yielded identical results, no particular recommendations for this particular test can be made.

TABLE 3. Assessment of DNase-detecting media

DNase medium (reference)	Result with NCTC strain[a]:				
	11351	11415	11637	12145	6571
Difco	−	−	−	(+)	+
Hänninen (5)	−	−	−	(+)	+
Holmes et al. (6)	−	−	(−)	(+)	+
Lior and Patel (8)	−	−	(−)	(+)	+

[a] −, negative result; (−), trace activity directly beneath inoculum, results obtained in two of three examinations only; (+), halo of activity extending ≤2 mm from the colony edge; +, halo of activity extending ≥4 mm from the colony edge. NCTC, National Collection of Type Cultures.

H_2S. Results for hydrogen sulfide production were generally as expected, and no differences in reproducibility were seen despite the use of inocula of differing densities. However, some strains always yielded irreproducible results (NCTC 11000, NCTC 11352, and NCTC 11415), so it may be prudent to exercise caution if this test is used to characterize the taxa that these strains represent. Either high (10^9 CFU/ml) or low (10^6 CFU/ml) inoculum densities can be used to inoculate the TSI medium.

Indoxyl acetate. The indoxyl acetate test was more reliable when disks impregnated with 25 μl of substrate were used instead of the 50 μl used by other workers. Discrimination between taxa was not compromised with the lower substrate volume. We therefore recommend the former substrate volume as a more prudent choice than the latter.

Nitrate reduction. Only Cook's method (3) yielded unequivocal results and a high (95%) level of reproducibility. The only strain to exhibit variable results with this method was NCTC 11000 (*C. mucosalis*). The other methods employed either did not discriminate between taxa (method of Itoh et al. [7]; all strains were positive) or were irreproducible (method of Cowan [4]; 25% of strains gave aberrant results). We therefore recommend Cook's method as the method of choice for determining nitrate reduction in campylobacteria, although care should be taken when examining strains purported to be *C. mucosalis*.

The results indicate that the methods used to detect certain enzymes useful for characterizing campylobacters should be carefully considered, since the ease of interpretation and reproducibility of such tests may vary considerably. However, the performance of certain enzyme tests, by the

methods described and recommended here, on these organisms can clearly provide rapid data for presumptive identification of a strain when these tests are used alone as well as when they are combined with other tests in a more conventional identification scheme.

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