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Reproducibility of Tolerance Tests That Are Useful in the Identification of Campylobacteria

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Twenty type or other reference strains, each representing a different *Campylobacter*, *Helicobacter*, or *Arcobacter* taxon, were used to assess the reproducibility of 25 phenotypic tests that are used in the identification of such organisms. Twenty-two of the tests depended on growth inhibition, and each of these tolerance tests was performed by using three different basal media. Although the overall reproducibility of the tests with each basal medium exceeded 89%, the proportion of strains that were able to grow in a reproducible manner on the basal media varied from 100% for blood agar and 50% for nutrient agar to 5% for brucella agar. In general, test reproducibility was highest with the basal medium that supported the most luxuriant growth. For the majority of tests, the basal medium which gave the optimum reproducibility could be determined.

The campylobacteria (the genera *Campylobacter*, *Helicobacter*, and *Arcobacter*) represent a taxonomically heterogeneous group, several species of which are important human and veterinary pathogens. The identification of campylobacteria is difficult since strains have relatively fastidious growth requirements and are asaccharolytic and since only a limited number of biochemical tests have been found to give adequate discrimination; for example, separation of *Campylobacter coli* from *Campylobacter jejuni* subsp. *jejuni* relies almost solely on hippurate hydrolysis. However, for both of these taxa, strains that do not give the expected result in this test have been reported (7). The development of reproducible tests that provide good discrimination for such taxa, and indeed, for the group as a whole, would therefore be of considerable value.

Resistance to various agents, temperature tolerances, and growth requirements are among the phenotypic tests used in characterizing campylobacteria. However, no standard methods for the performance of such tests have been published, and thus, most workers use methods peculiar to their own laboratory. Differences in methods may lead to differences in the outcome of, nominally, the same test. Certainly, there are such discrepancies published for campylobacteria. For example, the proportion of strains of *Helicobacter pylori* (formerly *Campylobacter pylori*) reported as tolerant to 1% glycine ranges from 0% (1) to 100% (4). Similarly, the proportion of strains able to grow at 42°C varies in *Helicobacter cinaedi* (formerly *Campylobacter cinaedi*) from 0% (3) to 20% (1) and in *Campylobacter fetus* subsp. *fetus* from 0% (9) to 59% (1). Furthermore, *Campylobacter mucosalis* was first described as being tolerant to salt (5), but the species description was later emended since salt tolerance was found to vary with the test method used (8).

It is well established in antimicrobial susceptibility testing that several factors may influence the outcome (10). These factors include the size of the inoculum and the composition of the basal medium. We have previously reported the influence of the former on phenotypic characterization of campylobacteria (6). The aim of the present study was to establish the possible effects on reproducibility of using three different basal media for tolerance tests.

MATERIALS AND METHODS

Bacterial strains. The 20 bacterial strains used in this study and their sources are listed in Table 1 (16 were type strains).

Media. The following basal media were used: (i) nutrient broth no. 2 (Oxoid Ltd., Basingstoke, England) with 5% (all percentages expressed as vol/vol or wt/vol where applicable) whole horse blood added after the autoclaved medium was cooled to approximately 50°C; (ii) nutrient broth no. 2 (Oxoid), unsupplemented; and (iii) brucella broth (Difco Laboratories Ltd., East Molesey, England), also unsupplemented. For all media, 2% Japanese agar was used as the gelling agent.

The following test media were prepared by previously described methods (6): 1% bile, 1% glycine, 2.0% NaCl, 0.1% selenite, 0.04% 2,3,5-triphenyltetrazolium chloride, and 0.1% trimethylamine *N*-oxide. The following test media were prepared by adding, aseptically, filter-sterilized solutions containing the appropriate amount of test substance to the autoclaved basal medium: 0.02% safranine O (BDH Ltd., Poole, England), 0.1% potassium permanganate (BDH), 0.02% sodium arsenite (BDH), and 5-fluorouracil (100 mg/liter; Sigma Chemical Co. Ltd., Poole, England). Carbenicillin (32 mg/liter), cefoperazone (64 mg/liter), cephalothin (32 mg/liter), metronidazole (4 mg/liter), and nalidixic acid (32 mg/liter) media were prepared by adding the appropriate number of ADATAB pellets (Mast Diagnostics, Bootle, England) to the basal medium, according to the instructions of the manufacturers. A campylobacter minimal medium (CMM) was prepared as described previously (6). A buffered charcoal-yeast (BCY) medium was prepared by the methods of Feeley et al. (2), but with 10 g of ACES buffer (Sigma) per liter added to maintain a stable pH. Preston basal (Campylobacter charcoal-desoxycholate [CCD]) medium (Lab-M, Bury, England) was prepared according to the instructions of the manufacturer. Growth at room temperature (ca. 18 to 22°C), 25, 30, 37, and 42°C (all under microaerophilic conditions) and growth under aerobic and anaerobic conditions at 37°C (25°C in the case of NCTC 12251) were determined on unsupplemented basal media. All strains grown at 37°C (25°C

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TABLE 1. Strains used in this study

Strain	Strain no.[a]	Source
Arcobacter cryaerophilus	NCTC 11885^T = CCUG 12012	Bovine, aborted fetus
Arcobacter nitrofigilis	NCTC 12251^T = CCUG 15893	Root of salt marsh plant
Campylobacter coli	NCTC 11366^T = CIP 7080	Pig feces
Campylobacter concisus	NCTC 11485^T = ATCC 33237	Human gingival sulcus
Campylobacter fecalis	NCTC 11415	Sheep feces
Campylobacter fetus subsp. *fetus*	NCTC 10842^T = CIP 5396 = ATCC 27374	Sheep fetus brain
Campylobacter fetus subsp. *venerealis*	NCTC 10354^T = ATCC 19438	Heifer vaginal mucus
Campylobacter hyointestinalis	NCTC 11608^T	Porcine intestine
Campylobacter jejuni subsp. *doylei*	NCTC 11951^T = IMVS 1141	Feces, 2-yr-old child
Campylobacter jejuni subsp. *jejuni*	NCTC 11351^T = CIP 702	Bovine feces
Campylobacter lari	NCTC 11352^T = ATCC 35221	Cloacal swab of herring gull
Campylobacter mucosalis	NCTC 11000^T	Porcine, small intestine
Campylobacter sputorum subsp. *bubulus*	NCTC 11367^T = CIP 53103	Bull sperm
Campylobacter sputorum subsp. *sputorum*	NCTC 11528^T = ATCC 35980 = CCUG 9728	Unknown
Campylobacter upsaliensis	NCTC 11540	Dog feces
Campylobacter spp., UPTC group	NCTC 11845	River water
Helicobacter cinaedi	NCTC 11611	Rectal swab, homosexual male
Helicobacter fennelliae	NCTC 11612^T = ATCC 35684	Rectal swab, homosexual male
Helicobacter mustelae	NCTC 12198^T	Ferret stomach
Helicobacter pylori	NCTC 11637^T	Human gastric antrum

[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, England; ^{T}, type strain.

in the case of NCTC 12251) under microaerophilic conditions on unsupplemented basal media served as controls with which growth on test plates could be compared.

Inoculum preparation. All bacterial suspensions were made in nutrient broth no. 2 (Oxoid Ltd.). For each strain, a suspension containing approximately 10^6 CFU/ml was prepared as described previously (6).

Inoculation and incubation of tests. Air-dried test plates were inoculated with 20 μl of the suspension delivered from a 50-dropper pipette. After allowing the spots to dry (not more than 15 min), all tolerance test, BCY, CCD, and CMM plates were incubated in anaerobic jars in which a microaerophilic atmosphere was created by reducing the atmospheric pressure to 540 mm/Hg (7.2×10^4 Pa) and regassing it with an anaerobic gas mixture (80% nitrogen, 10% hydrogen, 10% carbon dioxide). All tests were incubated at 37°C, except for those with strain NCTC 12251 (*Arcobacter nitrofigilis*), which were incubated at 25°C (the optimum growth temperature for this species), and for those to determine temperature tolerances, which were incubated at the appropriate temperatures. Each of the inoculated basal media was incubated aerobically to determine aerotolerance. Growth on trimethylamine *N*-oxide medium and that under anaerobic conditions were examined by incubating the appropriate media either in an anaerobic cabinet (Don Whitley Scientific Ltd., Shipley, England), in which the temperature was regulated at 37°C, or in an anaerobic jar, which was incubated at 25°C and in which the process of reducing the atmospheric pressure to 760 mm/Hg (1.0×10^5 Pa) and regassing with anaerobic gas mixture was performed twice (NCTC 12251 only). All tests were examined for up to 3 days, and each test was performed three times, on separate occasions and on freshly prepared media, in order to assess reproducibility.

RESULTS

The percentage of aberrance for each test and medium combination was calculated from the number of strains, of the 20 examined, which did not give a fully reproducible result (0 of 0 or 3 of 3 [Table 2]). At least one strain in 24 of the 25 tests demonstrated some variance, either in reproducibility or actual test outcome, between the basal media. This is illustrated in Table 3, which shows the number of positive results (of three) obtained in three example tests examined with each strain by using each basal medium. Tolerance to 1% glycine, for example, showed aberrance rates of 5, 25, and 20% when it was examined in blood, nutrient, and brucella agar media, respectively. The most reproducible results overall were obtained on blood agar (94.1% of results were reproducible), followed by nutrient agar (91.8%) and brucella agar (89.3%). These values were derived from Table 2 by first adding the percentage of aberrance values for each test (per basal medium), dividing the sum by the number of tests to obtain the mean percent aberrance per medium, and then subtracting the mean from 100%. Statistical analysis of the results (using a binomial factorial model adjusted for overdispersion) showed that the proportions of aberrant results between the basal media were significantly different ($P < 0.03$). The odds of an aberrant result occurring was found to be 1.45 times greater with nutrient agar and 2.00 times greater with brucella agar than they were with blood agar; the latter contrast was statistically significant ($P < 0.04$). On unsupplemented basal media and at their optimum growth temperatures, the best growth of strains was seen on blood agar (100% recovery); nutrient agar supported growth of 10 of the 20 strains (50%) reproducibly, but brucella agar supported growth of only 1 strain (5%) in a reproducible fashion (although recovery on this medium was improved under anaerobic conditions for certain strains). The percentage of fully reproducible positive results obtained was also calculated, to indicate the discriminatory power of each test and medium combination (Table 2). For example, no strains tolerated 1% bile with nutrient agar base (no discrimination), but 55% were positive with blood agar base (good discrimination).

TABLE 2. Percentage of aberrant and positive results obtained with 20 type or reference strains of campylobacteria in 22 tolerance tests by using three basal media, two charcoal media, and a minimal medium[a]

Test	Basal medium	Percent	
		Aberrance	Positive results
1% Bile	BA	5	55
	NA	0	0
	BrA	10	25
1% Glycine	BA	5	45
	NA	25	15
	BrA	20	20
Potassium permanganate	BA	25	40
	NA	5	25
	BrA	0	10
Safranine O	BA	25	70
	NA	10	35
	BrA	10	0
2% Salt (NaCl)	BA	5	30
	NA	0	10
	BrA	10	5
Selenite	BA	0	55
	NA	15	20
	BrA	5	10
Sodium arsenite	BA	10	15
	NA	0	20
	BrA	10	0
TMAO	BA	0	40
	NA	15	30
	BrA	20	25
TTC	BA	5	35
	NA	5	10
	BrA	5	10
Carbenicillin	BA	5	20
	NA	5	20
	BrA	5	5
Cefoperazone	BA	5	40
	NA	0	35
	BrA	5	5
Cephalothin	BA	0	40
	NA	15	15
	BrA	5	10
5-Fluorouracil	BA	5	65
	NA	5	50
	BrA	0	20
Metronidazole	BA	0	60
	NA	5	10
	BrA	5	0
Nalidixic acid	BA	0	45
	NA	20	20
	BrA	5	0
Growth at room temperature	BA	5	15
	NA	0	10
	BrA	5	5
Growth at 25°C	BA	5	20
	NA	0	20
	BrA	5	5
Growth at 30°C	BA	10	85
	NA	5	35
	BrA	15	5
Growth at 37°C	BA	0	95
	NA	40	45
	BrA	70	5
Growth at 42°C	BA	5	60
	NA	0	15
	BrA	15	0
Aerobic growth	BA	5	35
	NA	0	5
	BrA	5	0
Anaerobic growth	BA	5	35
	NA	10	40
	BrA	5	35
BCY		20	70
CCD		5	70
CMM		0	5

[a] BA, blood agar; NA, nutrient agar; BrA, brucella agar; TMAO, 0.1% trimethylamine *N*-oxide; TTC, 0.04% 2,3,5-triphenyltetrazolium chloride; BCY, buffered charcoal-yeast medium; CCD, campylobacter charcoal-desoxycholate medium (Preston medium base); CMM, campylobacter minimal medium.

DISCUSSION

The results indicate that the reproducibility of certain tests for certain taxa is questionable, even when the inoculum is standardized in terms of cultural age, optical density, and size and the tests are standardized in terms of atmospheric conditions and formulation. Test reproducibility proved to be dependent on the basal medium used, since no taxon gave irreproducible results in all three basal media for any of the tests examined (sample data shown in Table 3). When irreproducible results were observed, they were unlikely to be due to variations in the test media since a single batch of each dehydrate was used throughout the study. A more likely explanation is that each strain is so fastidious in its growth requirements that even minor variations of the test conditions affect the ability of the strain to grow. In general, the media giving the most favorable results for growth also yielded the most reproducible results overall (blood agar followed by nutrient agar followed by brucella agar). However, all three basal media gave an overall reproducibility rate of over 89% for the tests that were performed.

Blood agar would appear to be the medium of choice for most tolerance tests used in the identification of campylobacteria, provided that other important factors (such as the size of the inoculum and atmospheric conditions) are also considered. However, in some tests the use of a nutrient agar base gave results that were more reproducible (potassium permanganate, safranine O, sodium arsenite), and for

TABLE 3. The number of occasions on which growth (tolerance) was observed in certain tests used to characterize campylobacters when performed on three basal media

NCTC strain no.	No. of occasions growth was observed in the following tests on the indicated medium[a]:								
	1% Glycine			Sodium arsenite			Growth at 42°C		
	BA	NA	BrA	BA	NA	BrA	BA	NA	BrA
11351	3	3	3	2	3	0	3	3	1
11951	0	0	0	0	0	0	0	0	0
11366	3	3	3	1	0	0	3	3	2
11352	3	1	1	0	0	0	3	3	1
11845	3	1	0	3	3	0	3	0	0
11540	3	1	1	0	0	0	3	0	0
10842	3	3	3	0	0	0	3	0	0
10354	0	0	0	0	0	0	0	0	0
11608	0	0	2	0	0	0	3	0	0
11528	3	1	0	0	0	0	2	0	0
11367	3	1	3	0	0	0	3	0	0
11415	3	0	1	0	0	0	3	0	0
11485	1	0	0	0	0	0	0	0	0
11000	0	0	0	0	0	0	3	0	0
11612	0	0	0	0	0	0	3	0	0
11611	0	0	0	0	0	0	0	0	0
11885	0	0	0	3	3	1	0	0	0
11637	0	0	0	0	0	0	0	0	0
12198	0	0	0	0	0	0	3	0	0
12251	0	0	0	3	3	1	0	0	0

[a] BA, blood agar; NA, nutrient agar; BrA, brucella agar.

these it would seem prudent to use a nutrient agar base, particularly since little is lost in the discriminatory powers of these tests. There are also certain tests in which the level of test reproducibility was very similar between blood and nutrient agar bases (i.e., carbenicillin, cefoperazone, metronidazole), and further work involving a larger number of strains is required before recommendations can be made for a particular basal medium.

Any scheme for identifying a given group of organisms must be able to support their growth, particularly when the tests are of an inhibitory nature, in order to examine for true tolerance or susceptibility to the agent that is used. Difco brucella agar was clearly unsatisfactory in this respect, supporting fully the reproducible growth of only one strain. This differs from a previous study (6), in which five of the six strains were found to grow reproducibly on Difco brucella agar base; however, different atmospheric conditions were used in the present study. The observations that Difco brucella agar is unable to support the growth of many campylobacteria and that the quality of growth obtained in many of the taxa was more luxuriant on nutrient agar than it was on Difco brucella agar (as observed previously [6]) are unexpected since the latter is richer in nutrients than Oxoid nutrient agar is. Possibly, Difco brucella agar contains a substance that is inhibitory to the growth of campylobacteria, particularly under certain atmospheric conditions. No assumptions should be made of the likely effect that other brucella and nutrient agars might have on test outcome, because media composition may vary between manufacturers.

Most strains examined in this study were able to grow on the two charcoal-based media assessed (BCY medium and CCD [Preston charcoal base] medium), although the strains of *C. hyointestinalis*, *H. cinaedi*, *H. fennelliae*, and *H. pylori* exhibited growth only on the former medium and the strain of *A. nitrofigilis* exhibited growth only on the latter medium. Reproducibility also differed, with BCY showing a 20% aberrance rate compared with that of the Preston medium, in which only *C. concisus* failed to give reproducible results. Only *C. coli* grew on the minimal medium (CMM) and did so reproducibly.

The discrepancies reported in the literature for campylobacteria makes it difficult to compare test data gathered from different laboratories. The identification of campylobacteria is made particularly complex by such irreproducible characters; the effect of such a test on an identification may be diminished if a probabilistic scheme is used in which the failure of a taxon to perform reproducibly in a particular test can be accounted for by entering a probability value of 0.50. However, it should be noted that, provided that an appropriate basal medium was used, all the tests examined in the present study provided some level of discrimination between taxa. The use of a combination of these tests could therefore form the basis of an identification scheme, provided that the shortcomings of each test for each taxon is considered and the test methodology is standardized to as great a degree as possible.

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