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'1001' Campylobacters: cultural characteristics of intestinal campylobacters from man and animals

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SUMMARY

The cultural characteristics of 1220 *Campylobacter* strains from a variety of sources are described. Forty-two were identified as *Campylobacter fetus* ssp. *fetus* (Véron & Chatelain, 1973), 1120 as members of the *C. jejuni/C. coli* group, and 58 did not conform to any known description. Sixteen of the latter strains had the basic characteristics of *C. fetus* but were atypical in certain other respects. The other 42 strains had the thermophilic characteristics of the jejuni/coli group, but were resistant to nalidixic acid and had other features in common; it is possible that they represent a new species. They were isolated from 19% of locally caught wild seagulls but only occasionally from other animals and man.

Growth at 25 °C clearly distinguished strains of *C. fetus* from those of the jejuni/coli and the nalidixic acid-resistant thermophilic (NARTC) groups. Maximum growth temperature was less reliable for this purpose, and 43 °C was found to be better than the traditional 42 °C. By arranging the results of three tests (tolerance to 2,3,5-triphenyltetrazolium chloride, growth at 30·5 and 45·5 °C) serially in the form of a schema comprising nine categories, the jejuni/coli strains fell into two main groups resembling the Institute Pasteur *C. jejuni* and *C. coli* type strains, but these groups could not be clearly defined owing to the existence of strains with intermediate characteristics.

Most of the strains from cattle resembled *C. jejuni*, whereas those from pigs resembled *C. coli*; poultry strains occupied a more intermediate position. Strains from man and other animals were of mixed types, but most human strains resembled *C. jejuni* rather than *C. coli*. The type distribution pattern that most nearly matched that of human indigenous strains was given by a half-and-half mixture of strains from cattle and poultry.

INTRODUCTION

The recent emergence of campylobacters as a common cause of acute enteritis (Butzler *et al.* 1973; Skirrow, 1977), has created almost as many problems for the microbiologist as it has solved. Organisms that were virtually unknown in hospital laboratories have suddenly become commonplace – in 1979 over 8500 isolations were reported to the Communicable Disease Surveillance Centre, Colindale – yet we have no satisfactory method of classifying or typing them. Source tracing and other epidemiological investigations are therefore made difficult if not impossible, and it was to help remedy this state of affairs that we embarked upon the present work.

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Until the publication of the Approved Lists of Bacterial Names (1980) there were two classifications of campylobacters: the French (Véron & Chatelain, 1973) and the American (Smibert, 1974). This caused confusion. Almost all strains associated with acute enteritis belong to the group with thermophilic characteristics first described by King (1957) and referred to by her as 'related vibrios'. In the American classification all of these strains were included in a single subspecies (*C. fetus* ssp. *jejuni*), but in the French and currently accepted classification they are divided into the two species *C. jejuni* and *C. coli*, names derived from the *Vibrio jejuni* of Jones, Orcutt & Little (1931) isolated from calves, and the *V. coli* of Doyle (1948) isolated from pigs.

When Véron and Chatelain did their work, few strains were available for study, because good selective culture techniques were not generally available and there were difficulties over long-term preservation of cultures. The position has now changed, and the large collection of cultures that we have accumulated over the past few years provided, for the first time, a chance to study a wide range of organisms. This we have tried to do using simple methods to define broad groupings that might provide a basis for more specialized studies on selected strains.

MATERIALS AND METHODS

Source of strains

Table 1 lists the sources of the strains included in the study. In this laboratory 279 were isolated and 941 were received from other medical or veterinary laboratories, usually for confirmation of identification or in connexion with suspected outbreaks of infection. Of the human strains, 384 were classed as indigenous, i.e. they were isolated from patients who were not known to have been abroad within two weeks of becoming ill, 90 strains were isolated in Belgium, 47 in the Gambia (Billingham, 1980, in preparation), and 43 were isolated in other countries or from patients who had just returned from abroad; no information was available for 36 strains.

Most of the monkeys and baboons were recently imported animals, many of which were suffering from diarrhoea (Tribe, Mackenzie & Fleming, 1979). The seagulls were sampled as part of a survey of overwintering birds feeding on inland rubbish tips.* The Dunlin were caught on mud flats of the Severn estuary in South Wales.

The following strains were obtained from culture collections (these are included in Table 1):

National Collection of Type Cultures (*NCTC*), *Colindale, London*

5850	*C. fetus*	Milton. T. Dalling, Cambridge, 1939. Contagious abortion, sheep.
10842	*C. fetus* ssp. *fetus*	Mouton. I. R. Chatelain, 1972. Sheep fetus brain. This strain, originally isolated in 1952, is also in the Collection of the Pasteur Institute (CIP 5396) and in the American Type Culture Collection, Rockville, Maryland (ATCC 27374).

* Worcestershire and Warwickshire Wintering Gull Project. Organizer: G. H. Green, Windy Ridge, Little Comberton, Pershore, Worcestershire.

Table 1. *Source and identification of* Campylobacter *strains*

Source	No. of strains	Sample	*C. fetus* ssp. *fetus*	Atypical '*C. fetus*'	*C. jejuni*/ *C. coli*	Nalidixic acid-resistant thermophilic (NARTC)
Man	600	Faeces	1	0	564	2
		Blood	7	1	18	0
		Other	1*	0	6†	0
Cattle	76	Faeces/ intestine	7	11	55	0
		Products of conception	3	0	0	0
Sheep	105	Faeces/ intestine	2	2	65	0
		Products of conception	19	0	17	0
Pigs	95	Faeces/ intestine	2	2	90	0
		Products of conception	0	0	1	0
Dogs	67	Faeces/ intestine	0	0	61	6
Monkeys and baboons: *Macaca fascicularis*, *M. rhesus*, *Erythrocebus patas*, *Papio hamadryas*	55	Faeces/ intestine	0	0	51	1
		Products of conception	0	0	3	0
Cats	7	Faeces/ intestine	0	0	5	2
Horses	2	Faeces	0	0	2	0
Rodents: *Clethrionomys glareolus*, *Rattus norvegicus*	2	Faeces	0	0	2	0
Mink	1	Products of conception	0	0	1	0
Poultry	76	Faeces/caecum/ viscera	0	0	74‡	2
Seagulls: *Larus argentatus*, *L. fuscus*, *L. ridibundus*	59	Cloacal swabs	0	0	33	26
Dunlin: *Calidris alpina*	18	Cloacal swabs	0	0	18	0
Miscellaneous birds	31	Faeces and cloacal swabs	0	0	30	1
Environment	26	Water (reservoirs, pools and streams)	0	0	24	2
Total	1220		42	16	1120	42

* Tubo-ovarian abscess.

† Cerebro-spinal fluid (3); bile (2); urine (1).

‡ Includes four strains isolated in 1976 by J. E. Wilson & R. H. Duff (NCTC field strains A53/67; A55/67; A56/67; A58/67).

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10983	*C. fetus* ssp. ?	McCallum 89067. A. M. M. Wilson, 1975. Blood culture human.
11168	*C. jejuni**	5636/77 Lucitt. M. B. Skirrow. Faeces, human enteritis.
11353	*C. coli*	4620/78 Allsup. M. B. Skirrow. Pig placenta.
11352	*Campylobacter* sp.	3034/77 M. B. Skirrow. Herring gull (*Larus argentatus*).

Collection of the Pasteur Institute (CIP), Paris

702	*C. jejuni*	Uccle 91 F.B. A. Florent, Brussels. Bovine faeces.
7080	*C. coli*	1407. A. Florent, Brussels. Pig faeces.

Selection of strains

Where there appeared to be a single strain causing an outbreak, or a strain that was common to several members of a single group of animals, only one representative culture was included in the analysis.

Cultural methods

All strains isolated in this laboratory from faeces, and most of those received from other laboratories, had been isolated on selective agar containing vancomycin, polymyxin B, and trimethoprim (Skirrow, 1977). Cultures were checked for purity and propagated on blood agar (Oxoid B.A. Base No. 2 with 5% defibrinated horse blood) at 37 °C. Colonial morphology was noted. Cultures were preserved either in liquid nitrogen or at −20 °C in FBP broth containing 15% (v/v) glycerol (FBP broth: Oxoid Nutrient Broth No. 2 (CM67) containing 0·12% agar, and 0·05% each of $FeSO_4.7H_2O$, sodium metabisulphite and sodium pyruvate).

Atmosphere

Except where indicated cultures were incubated in anaerobic jars (without catalyst) from which two-thirds of the air had been withdrawn (500 mmHg below atmospheric pressure) and replaced with a 15% carbon dioxide/85% hydrogen mixture.

Tests

Tests were of three kinds: biochemical, growth temperature, and tolerance to certain chemical and antimicrobial compounds. Oxidation or fermentation of glucose was tested for by the method of Hugh & Leifson (1953), and nitrate reduction by the blood-agar plate method of Cook (1950). All other tests were done on agar plates whereby eight tests and two controls were accommodated on each plate (Fig. 1). The basal medium was Oxoid B.A. Base No. 2. (CM271) with the agar concentration increased to 2%; initially it was supplemented with 0·02%† sodium mercaptoacetate (thioglycollate), but later with the iron/bisulphite/pyruvate (FBP) supplement described by George *et al.* (1978). The concentrations

* Formerly designated '*C. coli* (provisional)' when originally deposited.

† This is a nominal figure, as each batch was titrated to determine the optimum concentration.

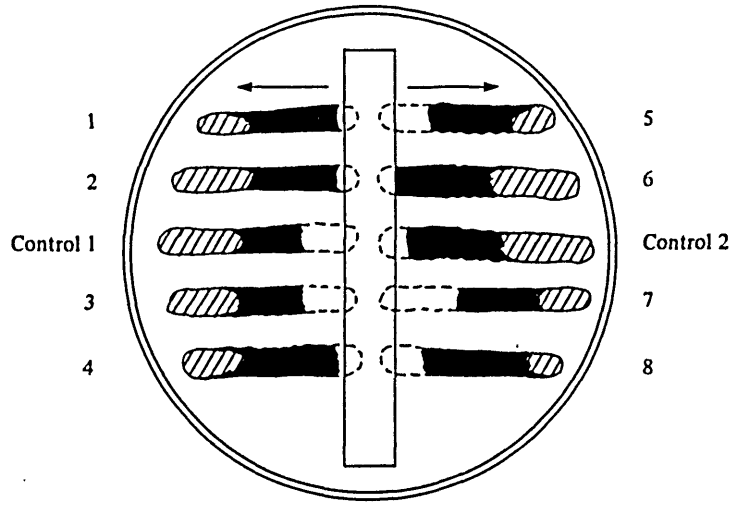

Fig. 1. Example of test plate (tracing of actual TTC test). Arrows indicate direction of spread of inoculating swab. Areas shown black are where tetrazolium has been reduced to the red formazan. Control 1 and test strains 3 and 5 are *C. jejuni*; control 2 and strains 1, 2, 4 and 6 are *C. coli*; strain 8 is an 'intermediate' strain and strain 7 is *C. fetus*.

finally used were 0·05 % of each compound as recommended in a later publication (Hoffman, Krieg & Smibert, 1979*b*) and as given in the FBP broth formula above. Plates were poured on the day of the test, or else poured on the previous day and stored under anaerobic conditions until used; they were protected from strong light.

Inoculum

The growth from blood agar cultures incubated overnight at 37 °C was suspended in FBP broth to a turbidity equivalent to Brown's tube No. 8. A swab charged with the suspension was touched on the side of the tube to drain excess fluid and then drawn rapidly and evenly across the designated area on each test plate (Fig. 1); the swab was re-charged for each inoculation of a given strain. Unless otherwise stated, all tests were incubated at 37 °C for 42 hours.

Controls

1. *C. jejuni*. NCTC 11168.
2. *C. coli*. NCTC 11353.
3. *C. fetus* ssp. *fetus*. NCTC 5850 (included on all 25 °C test plates and on other tests when comparisons with *C. fetus* strains were made).

Microscopic morphology

Smears made from the agar cultures were stained with 2 % carbol fuchsin for five minutes. Selected strains were examined with the wet flagellar mordanting stain of Mayfield & Inniss (1977).

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Table 2. *Concentrations of test substances used in strip and disc diffusion tests*

Test substance	Weight of paper* (g/m²)	Concentration of solution (g/l)	Content of disc (μg)
Nalidixic acid	140	0·06†	30
TTC (2,3,5-triphenyl-tetrazolium chloride)	300	40	—
Sodium hydrogen selenite	140	100	—
Metronidazole	140	0·1	5

* Ford's Gold Medal Blotting.
† Conveniently prepared from 10% solution in N/1 sodium hydroxide.

Oxidase

Growth taken from the agar cultures was smeared with a platinum wire on filter papers impregnated with 1% aqueous tetramethyl-*p*-phenylenediamine and previously dried. A positive test was indicated by a deep purple colour appearing within 10 seconds.

Catalase

A drop of 5% hydrogen peroxide was applied to the lawn of growth of each strain. Reactions were visually graded according to the amount of effervescence produced.

Selenite reduction

Impregnated blotting paper strips (Table 2) were applied to the basal medium as described for the tolerance tests (see below). Reduction of selenite was indicated by the production of bright orange-coloured selenium.

Growth temperature

Plates were incubated at 25, 30·5, 37 (control), 43 and 45·5 °C. 30·5 °C was the temperature at which Control 1 just failed to grow, and 45·5 °C the temperature at which the same strain grew only with difficulty. Jars were placed in ordinary incubators for the 37 and 43 °C tests, but they were placed in deep water baths for the 25, 30·5 and 45·5 °C tests. Actual temperatures were recorded with a mercury thermometer of certified accuracy (± 0·2 °C). The 25 and 30·5 °C tests were incubated for four days instead of two.

Tolerance tests

Strips cut from blotting paper known to be free from inhibitors were sterilized by autoclaving, and then soaked in a solution of the relevant compound and dried on plate glass in a drying cabinet (Petri dishes were unsuitable because of uneven drying). In some cases commercial discs (Mast Laboratories Ltd) were used as alternatives to strips. The substances used and their concentrations are shown in Table 2. Zones of inhibition to the edge of the strip or disc were measured to the

nearest 0·5 mm with a graduated caliper by a single observer. Measurement was made to the point at which the main growth ceased; any single colonies or thin tails of growth were disregarded. Corrections were applied if the zone sizes of the control strains on a given plate fell outside their mean values. Salt tolerance was measured by testing for growth on basal medium containing 1·5 and 3·5 % NaCl.

RESULTS

Basic identification of strains

All strains tested were Gram-negative spirilla or vibrios, all were oxidase- and catalase-positive, and 66 selected strains representing each of the groups and categories described below reduced nitrate to nitrite and failed to oxidize or ferment glucose. According to the criteria of Véron & Chatelain (1973), 1120 strains belonged to the *C. jejuni/C. coli* group, 42 strains were *C. fetus* ssp. *fetus* (including NCTC 10842 and NCTC 5850), and 58 were indeterminate. There were no strains of *C. fetus* ssp. *venerealis*. On the basis of temperature requirements and morphology, 16 of the indeterminate strains were deemed to be variants of *C. fetus*, and 42 shared the high growth-temperature characteristics of the jejuni/coli group, but differed in being resistant to nalidixic acid and possessing certain other common characteristics described below; these will be referred to as nalidixic acid-resistant thermophilic campylobacters (NARTC). The distribution of these groups according to their sources are shown in Table 1. All seven of the human blood isolates of *C. fetus* ssp. *fetus* were from patients with intercurrent disease or immune deficiency.

Colony morphology

C. fetus ssp. *fetus* strains produced small, round, convex colonies that grew to 1–2 mm in 48 hours at 37 °C. The 16 atypical strains produced basically similar types of colony, but three produced lemon-yellow pigment and two others – both isolated from cattle faeces – caused greening of blood agar.

Colonies of jejuni/coli group organisms were typically flat, becoming low convex, glossy, and effuse; large islands of growth were commonly formed, and many strains showed frank swarming on moist agar. Occasionally, a strain threw off discrete colonies, so that two colony types co-existed in one culture. On media containing high agar concentrations all strains produced a discrete type of colony resembling *C. fetus* ssp. *fetus*, hence the use of 2 % agar in the test media in order to prevent swarming (strains grew on medium containing even 6 % agar). Effuse and swarming growth was more characteristic of strains resembling the Institut Pasteur *C. jejuni* type strain than the *C. coli* type strain. In 18–24-hour cultures, colonies were almost transparent and resembled droplets of water sprayed on the medium. With continued incubation growth thickened and most strains took on a tan colour. Mature cultures of *C. jejuni*-like strains developed a 'metallic' surface sheen not unlike that seen on cultures of *Pseudomonas aeruginosa*; this feature was absent or much less evident with *C. coli*-like strains.

Microscopic morphology

C. fetus ssp. *fetus* organisms were generally larger than those of the jejuni/coli group. Long forms had regular open undulations with a long 'wavelength'. This meant that short forms were often only half a wavelength or less long and appeared as curved bacilli (vibrios). In contrast jejuni/coli group organisms were smaller and more tightly spiral, so that short forms appeared S-shaped. In general, bacterial cells of *C. jejuni*-like strains were smaller and finer than those of *C. coli*-like strains, but differences were slight. *C. fetus* ssp. *fetus* cells were usually more blunt-ended than those of the jejuni/coli group, which had tapering, indistinct ends. Flagellar staining showed *C. fetus* ssp. *fetus* to be predominantly monotrichate and organisms of the jejuni/coli group to be amphitrichate. *C. fetus* ssp. *fetus* organisms that were apparently amphitrichate could usually be seen to consist of a dividing pair of monotrichate cells. A strain of *C. jejuni*, isolated from the CSF of a 12-day-old baby, was persistently non-flagellate, although electron microscopy showed the presence of polar structures that are normally associated with flagellar insertion (Thomas, Chan & Ribeiro, 1980).

Cell morphology varied according to cultural conditions. Short, highly motile organisms predominated in young log-phase cultures; longer, less actively motile organisms were seen in more mature cultures. Coccoid forms were almost invariably present in post-mature cultures of the jejuni/coli group, but they were uncommon in cultures of *C. fetus* ssp. *fetus*. Small cocci were particularly common in cultures of *C. jejuni*-like strains, and large motile cocci in strains of the NARTC group. Predominantly coccal cultures subcultured only with difficulty or not at all. Some strains produced straight bacillary forms after they had been repeatedly subcultured.

Biochemical tests

Oxidase and catalase

All strains were oxidase- and catalase-positive. No attempt was made to quantify oxidase activity, but simple grading of the catalase test showed that 30 of 33 strains of *C. fetus* ssp. *fetus* gave strong reactions relative to strains of the jejuni/coli group. There was a wide range of activity in the latter group, but on average *C. jejuni*-like strains were less active than *C. coli*-like strains. On two occasions *C. jejuni*-like strains were referred to us as catalase-negative *Campylobacter* spp.

Selenite reduction

All strains tested reduced selenite. All but one of 39 strains of *C. fetus* ssp. *fetus* tested had low activity relative to strains of the jejuni/coli group which invariably produced a brilliant orange colour. Eight of 11 atypical *C. fetus* strains also showed low activity. These tests were performed on thioglycollate agar. When tests were re-run on FBP agar it was found that reduction of selenite was greatly diminished and the *C. fetus* strains were negative.

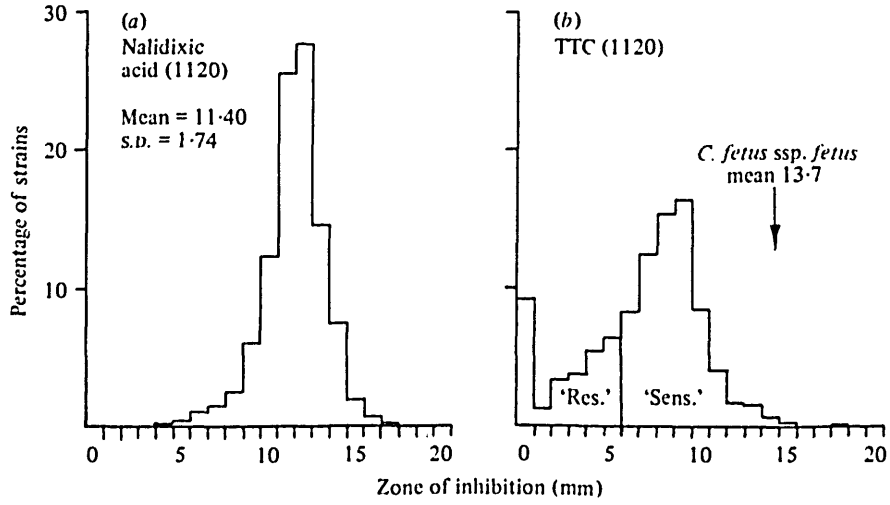

Fig. 2. Nalidixic acid and TTC tolerance of 1120 jejuni/coli strains.

Growth-temperature tests

All 42 strains of *C. fetus* ssp. *fetus* and the 16 atypical *C. fetus* grew at 25, 30·5 and 37 °C. All of 17 *C. fetus* ssp. *fetus* strains tested grew at a water-bath controlled 42 °C, but only one of 42 grew (slightly) when tested at 42·7 °C. However, six of eight atypical *C. fetus* strains tested grew in the 42·7 °C water-bath test.

In contrast, none of the *jejuni/coli* group or NARTC strains grew at 25 °C, and most of the former failed to grow at 30·5 °C. All grew more strongly at 43 °C than at 37 °C, but not all grew at 45·5 °C. The ability to grow at 30·5 °C or 45·5 °C was used in conjunction with TTC tolerance to classify strains according to a schema (see below).

Tolerance tests

Nalidixic acid

All 42 strains of *C. fetus* ssp. *fetus* were resistant and grew right up to the impregnated strip. This was equivalent to growing on agar containing 40 mg/l nalidixic acid (Véron & Chatelain, 1973). Four of the 16 atypical *C. fetus* strains showed intermediate sensitivity.

Of the thermophilic strains, 42 were not inhibited at all and constituted the NARTC group. All jejuni/coli strains were sensitive and gave a mean zone size of 11·40 mm (Fig. 2(*a*)). Six of the 18 strains from Dunlin gave reduced zones of 4·5–6·5 mm.

2,3,5-*triphenyltetrazolium chloride* (*TTC*)

All 42 strains of *C. fetus* ssp. *fetus* were sensitive to TTC (mean zone size 13·7 mm, S.D. 2·77). Most of the 16 atypical *C. fetus* strains were equally sensitive, but five gave reduced zones (minimum 4·5 mm). The jejuni/coli group showed a wide range of tolerance (Fig. 2(*b*)), and the fact that zones were usually clear and reproducible meant that this test was one of the most useful for differentiating strains within the group (see below). The NARTC strains were consistently sensitive (mean zone 10·0 mm, S.D. 2·67).

Sodium chloride

Strains of the jejuni/coli group were the most salt-sensitive. Excluding Dunlin strains, only 12 of 100 representative jejuni/coli strains grew on 1·5% NaCl agar. Of 16 Dunlin strains tested 14 grew on this medium and some of these showed reduced sensitivity to nalidixic acid (see above). All of 27 NARTC strains tested grew on the 1·5% medium, as did all but one of 19 *C. fetus* ssp. *fetus* strains. None of the strains that were positive on the 1·5% medium grew on 3·5% NaCl agar.

Metronidazole

In general, *C. fetus* ssp. *fetus* strains were resistant. Thirty-six of the 42 strains grew up to the strip or disc, though most showed some thinning of growth; the maximum zone size amongst the other six was 4·5 mm. All of the 42 NARTC strains were resistant.

The jejuni/coli group showed a wide range of sensitivity, but strains could be divided broadly into those showing no inhibition and those showing zones of more than 5 mm. Many of the sensitive strains formed a few colonies of resistant organisms sparsely and evenly distributed within the zones of inhibition, particularly when a heavy inoculum had been applied. The ratio of resistant to sensitive strains differed greatly in relation to their origin (Fig. 3(*a–i*)), but not in relation to schema category.

The main distinguishing features of the various campylobacter groups are summarized in Table 3.

Differentiation and distribution of thermophilic strains

Three of the most discriminatory tests (TTC tolerance, growth at 30·5 and 45·5 °C) were arranged sequentially to include all possible combinations (Table 4). In the temperature tests the simple criterion of the presence or absence of growth was used, except in the case of categories 2 and 3 which were separated on the grounds of graded growth at 45·5 °C. For TTC tolerance, strains giving zones of 6 mm or more were classed as 'sensitive' and less than 6 mm as 'resistant' (previous analysis had shown that this point gave the best separation relative to the other tests). Thus, a schema comprising nine categories was formed. Control strain 1 (NCTC 11168) belonged to category 2, and Control 2 to category 8. When the Institut Pasteur strains were tested – long after the scheme had been in use – *C. jejuni* (CIP 702) was found to belong to category 1, and *C. coli* (CP 7080) to category 8 like our Control 2. Thus, NCTC 11168 is a *C. jejuni*-like strain and its provisional 1977 designation as *C. coli* in the Type Collection has had to be amended. NCTC 10983 was found to belong to category 2, as did eight of the 17 other human strains isolated from blood; this was a higher proportion of category 2 strains (50%) than among the human faecal isolates (20%). Apart from this, disease severity did not appear to be associated with strain category: quite severe attacks were seen with *C. coli*-like strains as well as *C. jejuni*-like strains.

No major differences were observed between strain categories among the exotic

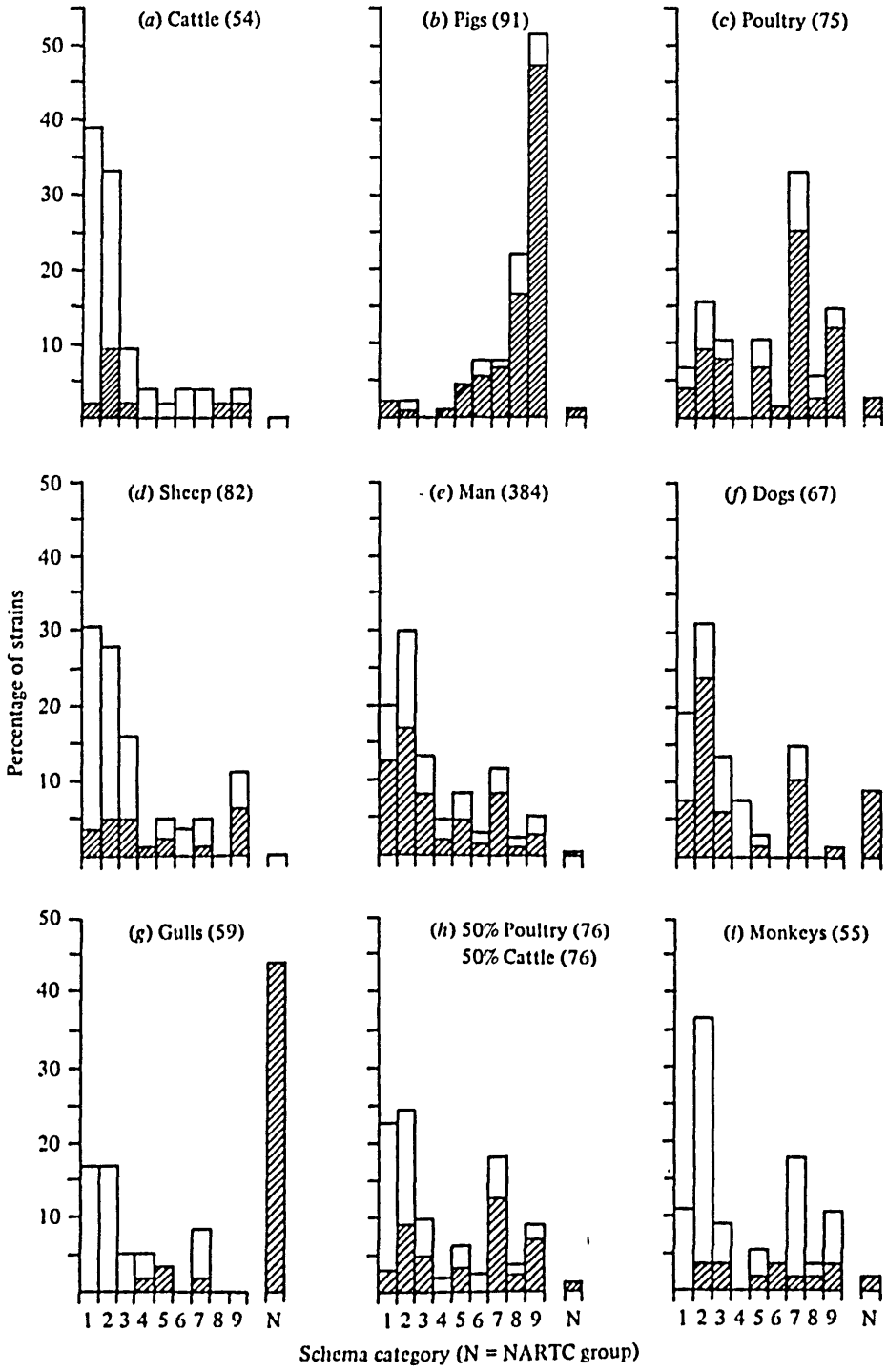

Fig. 3. Distribution of thermophilic campylobacter strains according to source. Figures in parentheses indicate number of strains tested. Hatched areas represent metronidazole-resistant strains.

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Table 3. *Main distinguishing features of intestinal campylobacters*

	C. fetus ssp. *fetus* (Control NCTC 5850)	*C. jejuni* (Control NCTC 11168)	*C. coli* (Control NCTC 11353)	NARTC* (Type strain NCTC 11352)
Morphology ...	Long wavelength, monotrichate	Short wavelength, amphitrichate	Short wavelength, amphitrichate	Short wavelength, amphitrichate
Coccal forms ...	Scarce	Common	Common	Very common
Colony type ...	Discrete	Effuse, spreading	Semi-effuse	Semi-effuse
Temperature (°C)				
25	+	−	−	−
30·5	+	−	+	+
37	+	+	+	+
43	−	+	+	+
45·5	−	Var.	Var.	+
Nalidixic acid	R (0)	S (11·5)	S (11·5)	R (0)
TTC†	S (13·5)	S (8·5)	R (0–3)	S (8·5)
1·5% NaCl	±	−	−	+
Metronidazole	R (0)	Var. (0)	Var. (11·5)	R (0)

+, Growth; ±, reduced growth; −, no growth; Var., variable; S, sensitive; R, resistant.

* Nalidixic acid-resistant thermophilic campylobacter.

† 2,3,5-triphenyltetrazolium chloride.

Figures in parentheses indicate readings of control or type strain where the mode is variable (figures = zones of inhibition in mm).

Table 4. *Schema for division of* C. jejuni/coli *strains*

TTC*	Sensitive					Resistant			
30·5 °C	−			+		−		+	
45·5 °C	−	±	+	−	+	−	+	−	+
Category	1	2	3	4	5	6	7	8	9

+, Growth; −, no growth; ±, reduced growth. * 2,3,5-triphenyltetrazolium chloride.

organisms, but there were more *C. coli*-like strains in the Belgian collection (15%) than in the British (6%), and almost all the Gambian strains (93%) were sensitive to metronidazole compared with the British figure of 41%.

NARTC strains are grouped separately, but had they been included in the schema 79% of them would have appeared in category 5, i.e. they were sensitive to TTC and grew at both 30·5 and 45·5 °C.

Figure 3(*a–i*) shows how the strains of the different categories were distributed among various animals. There was some overlap in pattern between the 26 strains isolated from natural water and the human strains, but eight (30%) of the former belonged to category 5 which was poorly represented among the human strains.

DISCUSSION

Cultural methods

Campylobacters have long been recognized as difficult organisms to study in the laboratory, and we certainly found this to be the case. Most problems stem from their unusual sensitivity to oxygen even though it is essential for their growth. It has been customary, therefore, to add reducing substances, such as thioglycollate, to campylobacter media, even when cultures are incubated under microaerobic conditions. The addition of blood to media was found to be beneficial, but its colour and opacity precluded its use for some of the biochemical and tolerance tests. Results with thioglycollate medium without blood were variable, not only between batches of medium, but also between individual plates; certainly exposure of plates to air and light seemed to be detrimental. This meant that each plate had to be carefully controlled, and in the early part of the work many tests had to be repeated, hence our choice of a strip-diffusion method that permitted each test strain to act as its own growth control. A particular feature of campylobacter behaviour was an unpredictable discrepancy between the size of the inoculum and the resulting growth, so that on 'toxic' plates the threshold between an inoculum that produced confluent growth and one that produced no growth at all was narrow. These problems were mitigated by the introduction of the iron/bisulphite/pyruvate supplement (FBP) described by George *et al.* (1978), which is now known to act by quenching superoxide anions and other free radicals in the medium and not by any action on cellular metabolism (Hoffman *et al.* 1979*b*; Hoffman *et al.* 1979*a*). Even with FBP-supplemented medium some variation remained, and this required that tests were fully controlled on medium that was freshly prepared and protected from strong light.

Differentiating tests

We confirm the value of temperature tolerance, first described by King (1957), in the classification of campylobacters. In this series the 25 °C test gave 100% separation between *C. fetus* and the jejuni/coli group. The highest temperature at which growth occurs is a critical one that requires control in a water bath with thermometers of certified accuracy. Even then results are likely to differ between laboratories according to variation in media, atmosphere, and size of inoculum; but it seems that 43 °C may well be a more suitable temperature than 42 °C for distinguishing between these species. Nalidixic acid sensitivity, while still a useful test in this respect, can no longer be regarded as infallible because of the existence of the NARTC group. We confirm that TTC tolerance is also useful for this distinction, but there was slight overlap between the more TTC-sensitive jejuni/coli strains and *C. fetus* ssp. *fetus*.

TTC tolerance was found to be particularly valuable for distinguishing strains within the jejuni/coli group, indeed the only other tests of merit in this respect were the tests for growth at 30·5 and 45·5 °C. Tolerance to 8% glucose and brilliant green, both quoted by Véron and Chatelain, were discarded as unhelpful

early in the work: the glucose test proved unreliable and only a narrow range of sensitivity to brilliant green was found among 352 jejuni/coli strains (unpublished). Tests for glycine tolerance were similarly found to be unhelpful. Tests for H_2S production have also been used to differentiate campylobacters and all the strains in this study were so tested, from which it was clear that *C. coli* strains were more active than *C. jejuni* and *C. fetus* ssp. *fetus* strains; however, tests were difficult to standardize and quantify and so they were not included in the analysis. Salt tolerance (1·5%) appears to be useful for helping to distinguish NARTC strains from the jejuni/coli group.

Metronidazole sensitivity is unusual in an organism that is not dependent on anaerobic metabolism. Whatever the mechanism involved, there is clearly a wide range of sensitivity among jejuni/coli strains (also found by Vanhoof *et al.* 1978) that is not shared by *C. fetus* ssp. *fetus* and NARTC strains. Resistance was not associated with any one schema category, but large differences existed between strains from different animal hosts, for example cattle (16% resistant) and pigs (85% resistant). The high resistance rate in pigs may be due to contact with nitroimidazoles which are widely used in intensive rearing systems in Britain.

Taxonomic implications

Differences between strains of *C. fetus* and strains of the *C. jejuni/C. coli* group, which were evident in almost every test, support the adoption of the French classification for the Approved Lists of Bacterial Names (1980). Although the status of *C. jejuni* and *C. coli* may be less sure, the differences that undoubtedly exist between these two, and the existence of the NARTC group make it inconceivable that all of these thermophilic organisms should be included in a single subspecies (*C. fetus* ssp. *jejuni*) as in the American classification. It remains to be seen whether the strains with characteristics intermediate between *C. jejuni* and *C. coli* represent yet other groups.

The NARTC strains form a well-defined group that, according to Gordon's definition, might warrant species status (Gordon, 1978); apart from their resistance to nalidixic acid, they are salt tolerant relative to the jejuni/coli group, are sensitive to TTC, resistant to metronidazole, have a wider range of growth temperature, and readily form coccoid bodies. The first four features are like those of *C. fetus*, but the last two are in sharp contrast to them. Strains from Dunlin seem to occupy a position intermediate between the NARTC and orthodox jejuni/coli strains.

The atypical strains that we regard as belonging to the species *C. fetus* are a miscellaneous group, but it may be noted that some were able to grow at 43 °C and therefore might be confused with the thermophilic group if not tested at 25 °C.

Epidemiological implications

The distribution of jejuni/coli strains, according to category, among the various animal species make interesting comparison (Fig. 3*a–i*). Dissimilar histograms indicate that strains are not shared. For example, in Britain, it seems that pigs do not contribute more than a small proportion of human infections, and it is clear

that no one animal species is the source of all human infections. Similar histograms, on the other hand, can indicate only possible epidemiological links. The similarity of indigenous human strains with a half-and-half mixture of cattle and poultry strains is in keeping with what little we know of the epidemiology of campylobacter enteritis, but it would be absurd, for example, to read significance into the similarity with the monkey histogram since both hosts probably derive infections from similar sources. The dog histogram may bear an unrepresentative resemblance to the human one, because about a third of the dog strains were obtained through follow-up of human patients. Dogs were the only animals to have more than a very low percentage of NARTC 'seagull' strains. It is interesting that the strains from gulls that were not NARTC strains belonged mainly to categories 1 and 2, like those predominant in cattle and sheep. This raises the possibility that gulls, by roosting and loafing on pastures, contribute to the reservoir of infection in farm stock, and since some of these strains can cause ovine abortion, this could have important economic consequences.

The allocation of strains into categories has also proved useful for comparing isolates implicated in outbreaks of human infection. Direct comparison makes it possible to detect more subtle differences between strains even of the same category, and thus to recognize epidemic strains. This has proved its worth on several occasions, e.g. Jones *et al.* (1980, in preparation). But the fact remains that there is still no generally applicable method of classification or strain identification for enteropathogenic campylobacters. We hope that these results will provide a basis from which others can work towards this goal.

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