IDENTIFICATION AND TYPING OF BACTERIA

Application of the Mast resistotyping scheme to *Campylobacter jejuni* and *C. coli*

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The Mast resistotyping scheme was assessed with 228 strains of *Campylobacter jejuni* and *C. coli* from enteric infections in man and from a diverse selection of other sources (livestock, chickens and river water). Most (153 of 158) *C. jejuni* examined were of the three most common Penner (heat stable, HS) serotypes, HS1, HS2 and HS4 complex. Fourteen resistotypes were identified in the 158 strains of *C. jejuni* and 16 in the 70 isolates of *C. coli*. The predominant codes were 00 (44% of *C. jejuni*; 33% of *C. coli*) and 40 (21% of both species). The scheme was simple to use but reproducibility and interpretation of sensitivity zones – notably for fluorouracil, triphenyltetrazolium chloride and metronidazole – was occasionally problematic. Overall, resistotypes did not correlate with Penner HS serotypes or with three key genomic markers (ribotype, PFGE macrorestriction-type and *fla*-type). Although resistotyping offers a rapid means for distinguishing between some strains of *C. jejuni* and *C. coli*, discrimination for common resistotypes can be achieved only in combination with other typing methods.

Introduction

Most human cases of campylobacteriosis are sporadic infections which are believed to be caused by *Campylobacter jejuni* and *C. coli*, although clinical laboratories in the UK rarely speciate isolates [1]. Surveillance data on foodborne infectious intestinal disease generally treat all such strains simply as *Campylobacter* spp. [2]. Outbreak investigations demand more detailed identification, and several biotyping schemes have been developed over the past 15 years as cost-effective and convenient phenotypic methods for characterising thermotolerant campylobacters.

The various biotyping schemes developed for *C. jejuni* also provide a basis for subtyping *C. coli*. Four biotypes of *C. jejuni* and two of *C. coli* were defined in the widely used Lior extended scheme [3] and greater discrimination was achieved in the Preston scheme [4, 5] in which three biochemical and 10 resistotyping tests were used. The advantage of that scheme was that it enabled most clinically important campylobacters to be speciated and biotyped. In the present study, the reproducibility and discriminatory

power of the six-test Mast *Campylobacter* Biotyping Scheme developed as a simplification of the Preston scheme have been examined. Resistotypes were determined on a set of *C. jejuni* and *C. coli* isolates from human disease and from healthy animals. Discrimination was assessed by reference to Penner serotyping, and to genomic analyses based on previously published ribotyping and pulsed-field gel electrophoretic (PFGE) DNA macrorestriction profiles [6–8] together with *fla*-typing [9].

Materials and methods

Bacterial strains and growth conditions

The reference numbers and origins of the 158 strains of *C. jejuni* tested in this study were given previously with details on their Penner (HS) serotypes, 16S ribotypes and pulsed-field gel electrophoretic (PFGE) profiles [6–8]. Most strains were serotypes HS1 (52 strains), HS2 (36 strains) and HS4 complex (63 strains) and included 117 human isolates (41 HS1; 27 HS2; 44 HS4 complex; 2 HS11; 1 HS44; 2 HS53), 21 bovine isolates (7 HS1; 7 HS2; 6 HS4 complex; 1 HS8/17), nine dog isolates (1 HS1; 8 HS4), three chicken isolates (2 HS1; 1 HS12) and eight reference strains from the NCTC collection. Representative strains from two outbreak sets were examined and comprised five isolates (HS2)

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from a milk-borne outbreak in the Cardiff area [7] and four HS1 strains from a water-borne outbreak which occurred in the UK in 1988 [6]. The reference numbers and origins of the 70 strains of *C. coli* tested in this study were also given previously with details on their Penner serotypes, 16S ribotypes, flagellin gene (*flaA*) types and PFGE profiles [8].

Bacterial isolates, mostly collected in the UK between 1993 and 1995, were preserved over liquid nitrogen on plastic beads in Nutrient Broth No. 2 (Oxoid CM67) 1% w/v containing glycerol 10% v/v. Strains were subcultured initially on defibrinated horse blood agar 5% v/v for 24 h at 37°C under micro-aerobic conditions (CO₂ 5%, O₂ 5%, H₂ 2% and N₂ 88%) in a Variable Atmosphere Incubator (Don Whitley Scientific Ltd, Shipley, Yorks).

Resistotyping

The resistotyping scheme consisted of the Mast *Campylobacter* (Camp Bioring) biotyping ring and

Campylobacter biotyping agar (a blood-free charcoal medium). The components were purchased from Mast Diagnostics (Bootle, Merseyside). The biorings comprised six arms with tips impregnated with: tetracycline (T 18 µg); nalidixic acid (NA 30 µg); metronidazole (MZ 5 µg); 2,3,5-triphenyltetrazolium chloride (TTC 1000 µg); 5-fluorouracil (FUR 80 µg) and sodium arsenite (SAR 30 µg).

A suspension of the test organism (24 h growth at 37°C on biotyping agar) was prepared to a density equivalent to McFarland's Standard No. 2 in maximum recovery diluent (MRD) and then further diluted 1 in 100 in MRD. Biotyping agar plates were inoculated by flooding the surface with 1.5 ml of the diluted bacterial suspension. Each plate was drained of excess liquid and dried at room temperature. The rings were placed on the surface of each plate, which was incubated under micro-aerobic conditions at 37°C for 24 h. The inhibition zones were measured with calipers and results were scored according to the manufacturer's instructions. A proportion of cultures

Fig. 1. Distribution of isolates according to resistotype at 24 h: ■ *C. coli*, ▨ *C. jejuni*.

were then re-incubated for a further 24 h and zones were measured again.

Results

Resistotypes at 24 h

A total of 14 resistotypes was recorded at 24 h amongst the 158 strains of *C. jejuni* and 16 resistotypes amongst the 70 *C. coli* strains tested. The distribution of strains according to resistotypes is shown in Fig. 1. There were two predominant types for *C. jejuni* with codes 00 (44%) and 40 (21%), and three for *C. coli* with codes 00 (33%), 40 (21%) and 02 (11%). Code 00 strains were fully sensitive to all six agents whereas the isolates with code 40 were resistant to metronidazole and isolates with code 02 were resistant to 5-fluorouracil. Strains with other codes were rarer and each accounted for < 7% of strains. Five strains (2%) were non-typable as they did not grow within 24 h on *Campylobacter* biotyping agar when the inoculum was prepared according to manufacturer's instructions. When a higher inoculum was used (1 in 50 dilution of a bacterial suspension equivalent to McFarlands No. 2), results were obtained at 24 h for all four strains tested.

Two outbreak sets of *C. jejuni* strains were included in the study. Two of the four isolates from a water-associated outbreak (HS1) were code 00 and two were code 02, whereas the five strains from a milk-associated outbreak (HS2) were all code 00.

Resistotypes at 48 h

Results for all 70 *C. coli* strains were recorded at 48 h. Results for 42 *C. jejuni* isolates were also re-read at 48 h when 24-h results were equivocal because growth was either very light or just detectable within the zones causing them to be hazy and difficult to interpret. Sixty percent (42 of 70) of *C. coli* strains and 79% (33 of 42) of the *C. jejuni* examined had a different resistotype at 48 h from that recorded at 24 h. Results for the strains with changed resistotypes at 48 h are summarised in Table 1. Three new resistotypes (codes 46, 52 and 63) were observed at 48 h with *C. jejuni* and seven new resistotypes (codes 12, 15, 43, 46, 53, 56 and 62) were observed at 48 h with *C. coli*, but three codes were lost with *C. coli*. For many strains with altered resistotypes (49 of 75; 65%), the differences were attributed to changes in the FUR results.

Reproducibility of resistotypes at 24 h

The reproducibility of resistotyping results was investigated with a subset of 77 strains (41 from 158 of *C. jejuni* and 36 from 70 of *C. coli*). These strains were selected proportionally to represent the different resistotype codes encountered in the study. A total of 21 (51%) of 41 strains of *C. jejuni* and 31 (36%) of 36

C. coli strains had different initial and repeat codes as listed in Table 2. On repeat testing, four new codes (41, 46, 54 and 62) were gained and one was lost (74) for *C. jejuni*, and three new codes were gained (12, 15 and 43) and two were lost (06 and 11) for *C. coli*. The changes in resistotype seen in *C. jejuni* were often associated with FUR (52%; 11 of 21 strains) whereas

Table 1. Summary of results for strains of *C. jejuni* and *C. coli* with differing resistotypes at 24 h and at 48 h

Code		Number of strains		Agent associated with changed code
24 h	48	<i>C. jejuni</i>	<i>C. coli</i>	
00	01		1	TTC
00	02	19	14	FUR
00	04	1		SAR
00	40	2		MZ
00	42	1		FUR + MZ
00	46	2	2	MZ + FUR + SAR
02	07		5	TTC + SAR
04	06		2	FUR
06	07		1	TTC
10	12		2	FUR
11	15		1	SAR
40	41		2	TTC
40	42	2	3	FUR
40	43		4	TTC + FUR
40	46	2		FUR
40	60	1		NA
41	43		1	FUR
41	51		1	T
41	53		1	T + FUR
42	44	1		SAR + FUR
50	52	1		FUR
54	56		1	FUR
60	62		1	FUR
61	63	1		FUR

TTC, 2,3,5-triphenyltetrazolium chloride; NA, nalidixic acid; FUR, 5-fluorouracil; T, tetracycline; SAR, sodium arsenite; MZ, metronidazole.

Table 2. Summary of results for strains of *C. jejuni* and *C. coli* with differing resistotypes on repetition

Code		Number of strains		Agent associated with changed code*
Initial	Repeat	<i>C. jejuni</i>	<i>C. coli</i>	
00	01		2	TTC
00	02	5		FUR
00	40	1		MZ
00	42	1		FUR + MZ
02	00	1		FUR
02	04		1	SAR
02	07		1	TTC + SAR
04	44	1		MZ
06	05		1	TTC + FUR
10	12		1	FUR
11	15		1	SAR
40	41		4	TTC
40	42	4		FUR
40	44	2		SAR
40	46	1		FUR + SAR
41	43		1	FUR
41	51		1	T
60	62	1		FUR
70	71	1		TTC
71	40	1		T + NA + TTC
71	41	1		T + NA
74	54	1		NA

*Agents as listed for Table 1.

TTC-associated changes were most common in *C. coli* strains (46%; 6 of 13).

Associations between resistotype and serotype

Table 3 shows the distribution of *C. jejuni* and *C. coli* strains with respect to resistotypes (24-h result) and the main Penner serotypes studied (*C. jejuni*: HS1, HS2 and HS4 complex; *C. coli*: HS54, HS53, HS49 and HS46). Strains within each of the resistotypes were diverse with respect to species and serotype, with only four resistotypes and serotypes (10 strains) that were uniquely associated. Seven resistotypes were common to both species (00, 02, 04, 10, 40, 42 and 60).

Associations between resistotype and genotype

Strains of *C. jejuni* and *C. coli* within each resistotype (24-h result) were compared with previously published genotypic data [6–8]. All resistotype groups, with the exception of that coded 12 (two *C. jejuni* strains) were diverse with respect to PFGE type. Overall, PFGE based on *Sma*I digests of genomic DNA was the most discriminatory method; for example, for *C. jejuni*, 21 different profiles were detected amongst the 70 strains of resistotype code 00, and 17 amongst the 32 strains of code 40. However, the four isolates from a water-associated outbreak had identical PFGE and ribotypes, yet two of the strains were coded 00 and two were coded 02. There were 28 different PFGE profiles in the *C. coli* strain set with six profiles amongst the 23 strains of resistotype code 00, and nine different profiles amongst the 15 strains of code 40. Table 3

Table 3. The coincidence of resistotypes within clonal lines* in *C. jejuni* and *C. coli* at 24 h

Clone identity†	Number of strains	Mast resistotype (number of strains)
<i>C. jejuni</i>		
HS1 clonal line 1	10	00 (6), 02 (2), 40 (2)
HS1 clonal line 2	4	00 (1), 40 (2), 42 (1)
HS1 clonal line 3	3	00 (1), 40 (2)
HS1 clonal line 4	3	40 (2), 61 (1)
HS2 clonal line 1	7	00 (6), 40 (1)
HS2 clonal line 2	4	00 (2), 02 (1), 40 (1)
HS2 clonal line 3	4	42 (2)
HS4 clonal line 1	6	00 (4), 02 (1), 10 (1)
HS4 clonal line 2	6	00 (1), 02 (1), 40 (3), 42 (1)
HS4 clonal line 3	5	00 (5)
HS4 clonal line 4	4	40 (1), 44 (3)
HS4 clonal line 5	4	40 (1), 50 (3)
HS4 clonal line 6	3	00 (3)
<i>C. coli</i>		
HS54 clonal line	13	00 (10), 02 (2), 10 (1)
HS53 clonal line	7	00 (7)
HS49 clonal line	7	02 (5), 06 (1), 07 (1)
HS46 clonal line 1	4	40 (4)
HS46 clonal line 2	4	00 (4)

*Clonal lines defined on the basis of identity in serotype, ribotype, PFGE DNA profiles, and with the exception of *C. jejuni* HS2 strains, flagellin gene type [6–9].

†HS, serotype (heat stable antigen).

lists the resistotypes recorded at 24 h that were associated with each of the clonal lines described previously. These clones were defined on the basis of identity in serotype, ribotype, PFGE DNA macrorestriction profiles and, in some cases, flagellin gene type. Most clones were not homogeneous at 24 h with respect to resistotype.

Discussion

Since 1984, biotypes within *C. jejuni* and *C. coli* have been identified mainly by the Lior scheme [3] and by the more discriminatory Preston scheme [4, 5]. However, there are only limited data on the frequency and distribution of the biotypes in the UK [10] and the usefulness of biotyping for epidemiological purposes has not been clearly established. The Mast Campylobacter Biotyping Scheme is the first commercially available biotyping scheme for these organisms. The present results show that it was simple and fast to perform and gave a result for most strains within 24 h. Compared with Lior biotyping, it provided improved discrimination between strains and typability was high at 24 h. However, detailed analysis of the resistotyping results raised several important points relating to their interpretation.

First, the susceptibilities of some strains were difficult to score at 24 h and when this was due to poor growth or hazy zones there was a logical inclination to re-incubate the plates and to score results at 48 h. The study showed that the 48-h results provided no advantage in reproducibility or discrimination. In fact, it led to a shift in the spectrum of resistotypes, and a decrease in the overall level of discrimination for *C. coli* with code 02 becoming the single predominant type. From our strain set, it was clear that the FUR test was the most problematic of the agents and needs to be investigated with a larger number of both *C. coli* and *C. jejuni* strains of different serotypes. Poor growth at 24 h could be avoided, possibly by increasing the inoculum density, although the effect that might have on resistotyping results needs to be examined systematically. Thus culture conditions, such as the degree of dryness of biotyping agar plates, require careful monitoring because they may have a significant effect on the rate of growth.

Second, a lack of reproducibility was found in some instances when 24-h resistotyping results were repeated by the standard procedures. For 47% (16 of 34) of the retested strains the different code obtained on a single repetition was linked to the FUR test. The TTC results for *C. coli* also lacked reproducibility and were problematic for about one-third of strains. The results indicate that more extensive reproducibility studies are necessary to establish the reliability of the resistotyping scheme both within and between laboratories because of the difficulties of interpreting hazy zones.

Degree of discrimination is a key criterion for assessing the value of a typing scheme [11]. The overall degree of discrimination achieved with resistotyping was clearly better than that of other biotyping schemes, with 14 different resistotypes of *C. jejuni* and 16 of *C. coli* in 228 strains compared with just four and two, respectively, with the Lior scheme. Furthermore, comparison with other typing methods indicated that resistotyping can discriminate within serotypes and ribotypes. However, a feature of the data was the fact that the majority (65%) of the *C. coli* isolates belonged to just three resistotypes (00, 40 and 02) and 65% of strains representing the most common UK serotypes of *C. jejuni* belonged to two resistotypes (codes 00 and 40). Further *C. jejuni* isolates representative of other serotypes need to be tested to establish if relative homogeneity is a consistent feature of the typing scheme rather than a result of biased strain selection. Every effort was made in the present study to randomise the strains used as much as possible with respect to host, time and location of isolation. Only four other resistotypes (04, 10, 42 and 60) were common to both species, so there is a possibility that a proportion of resistotypes may be species-specific but more strains of *C. jejuni* need to be tested.

A desirable attribute of any typing scheme is that the types defined can be validated as far as possible by reference to other typing methods available for that micro-organism. Comparison of resistotyping data with Penner serotyping, an internationally recognised scheme, indicated considerable antigenic diversity within the predominant resistotypes. Similarly, comparison with genotypic data from ribotyping and PFGE profiling [6–8] also demonstrated a high level of intra-resistotype diversity. In contrast, four strains from a single outbreak which were identical with respect to serotype, PFGE profile and ribotype were divided into two groups by the resistotyping scheme, one coded 00 and the other coded 02. It should be noted that this difference in codes is based on the FUR result, which was shown to be the least reproducible test. Overall, the results showed that resistotyping was not validated by reference to the other three typing methods, and that resistotyping did not provide a logical and valid basis for defining meaningful subtypes within *C. jejuni* and *C. coli*. In practice, the Mast system can be applied to screen for strains that do not share the same resistotypes. Where there are reproducible differences, it provides a marker for isolates that may be epidemiologically unrelated. However, strains with the same resistotype need to be investigated further by serotyping or by molecular

typing to establish their affinities with more precision. For example, code 00 occurred in both outbreak sets of *C. jejuni* studied here yet the strains had different serotypes (HS1 and HS2) and could be further discriminated by PFGE profiling.

In conclusion, Mast resistotyping provides a simple and rapid approach to distinguishing between isolates within *C. coli* and strains of *C. jejuni* HS1, HS2 and the HS4 complex. However, as the system also groups apparently unrelated organisms, strains with the same resistotype must be subjected to further analysis by high resolution typing methods if the scheme is to be of value in future outbreak investigations. Factors that determine reproducibility of the test results need further evaluation, so that data from different laboratories can be compared with confidence.

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