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Resistotyping of campylobacters: fulfilling a need

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SUMMARY

A 9-month trial of a simple typing scheme for 'thermophilic' enteric campylobacter isolates at a large Public Health Laboratory is described. Resistotyping was performed with six agents in a method modified by Bolton and colleagues from an earlier scheme, and biotyping was performed by a modified Lior scheme involving three tests. Reproducibility was excellent in both schemes, with test variation $< 2\%$. Five household clusters and one larger presumptive milk-borne outbreak were identified in this scheme, and confirmed in pyrolysis mass spectrometry. The 328 isolates from new patients, excluding duplication from these clusters, were divided into 35 resistotypes with the largest group comprising 22% of isolates. In combined bio- and resistotyping, 86 types were found, with the largest group comprising 9·5% of isolates. The results are contrasted with salmonella sero- and phage-typing, where, on the same basis, the 176 isolates in the same period were divided into 40 groups, with the largest comprising 45% of isolates. Resistotyping, with or without additional biotyping, proved to be a convenient, simple, rapid, highly discriminatory, reproducible and inexpensive method well suited to use in local laboratories. It is a strong candidate for first-line national and local surveillance of campylobacter infections, fulfilling a need for monitoring of this important cause of enteric disease.

INTRODUCTION

Gastro-intestinal infection with *Campylobacter jejuni* and other 'thermophilic' campylobacters is a major public health problem in the UK and world-wide. The incidence of laboratory-proven infection exceeds that for salmonellae in the UK and is continuing to increase [1]. However, our knowledge of the epidemiology of campylobacter infection is poor, particularly when compared to that for salmonellae. The availability of suitable typing schemes is a key factor contributing to this stark contrast. O and H serotyping of salmonellae is a rapid, discriminatory method, sufficiently inexpensive and simple to be applied to all salmonella isolates. The widespread use of serotyping at a local level allows rapid response in the investigation of suspected outbreaks, and further discrimination is available from centralized phage typing schemes. Salmonella serotyping also shows good inter-laboratory reproducibility and is generally accepted, allowing country-wide surveillance. In contrast, there is no generally accepted, locally applicable scheme for campylobacter typing.

Various phenotypic and genotypic typing methods have been suggested for campylobacters, including biotyping [2–6], phage typing [7, 8], serotyping [9–12], SDS-PAGE of whole cell proteins [13], restriction fragment length polymorphisms [14], ribotyping [15–17], pulsed field gel electrophoresis [18], multi-locus enzyme electrophoresis [19] and randomly amplified

polymorphic DNA analysis [20]. None of these schemes possesses the combination of simplicity, reproducibility, speed and discrimination that would allow its routine use in local laboratories.

Approximately 400 'thermophilic' campylobacters are isolated each year at the Cardiff Public Health Laboratory (PHL) from human faecal samples. Until 1993, a small proportion of these isolates was biotyped in the abbreviated scheme of Skirrow and Benjamin [3]. This had proved to be of little discriminatory value; only four specific types were found and most strains were identified as *C. jejuni* biotype 1. A trial of a disk sensitivity resistotyping scheme which was developed at the Preston PHL was initiated in late 1993. This scheme was a modification of an earlier biotyping method [6] which had included patterns of resistance to various inhibitory agents. Preston PHL had found the new scheme promising, and it appeared to possess many of the properties required for local surveillance and fast-response investigation of outbreaks. A modified biotyping scheme [4] that might add discrimination for minimal expenditure was investigated concurrently. These two schemes were applied at the Cardiff PHL to isolates from all new cases of infection from January 1994. Isolates from suspected outbreaks and isolate clusters were also analysed in pyrolysis mass spectrometry (PMS) [21–23] which had been investigated previously as a fingerprint typing method for campylobacter and had yielded promising results (see below). This paper outlines our experience with this approach over the first 9 months of the trial.

MATERIALS AND METHODS

Bacterial strains

Routine faecal samples were cultured on Blood-Free Campylobacter Selective Agar (Oxoid) incubated microaerobically (10% carbon dioxide, 90% nitrogen) at 42 °C for 48 h. Presumptive isolates of 'thermophilic' campylobacters were confirmed by colonial morphology and Gram stain. A single well separated colony of each confirmed isolate was picked on to blood agar (5% horse blood, Tissue Culture Services, in Columbia Agar Base, Oxoid) incubated microaerobically for 24 h at 37 °C and a suspension of the growth was stored at −80 °C in glycerol broth. Isolates from all new cases of infection from January to September 1994 were resistotyped and biotyped in batches. In the course of the trial several family clusters and a suspected point source outbreak were identified; isolates from these were also investigated in pyrolysis mass spectrometry.

Resistotyping

Frozen isolates were recovered on blood agar, and a suspension of the growth equivalent to MacFarlane standard 2 was prepared in 5 ml of Brain Heart Infusion Broth (Oxoid) in a bijoux bottle. The caps were screwed tight, and the broth was incubated aerobically at 42 °C for 4 h. A well dried Columbia Agar Base (Oxoid) plate was flooded with the suspension, the excess was drained with a Pasteur pipette and the plates were left for *c.* 40 min to dry. Whatman 6 mm AA filter paper disks containing tetracycline hydrochloride (Tet; Sigma; 3 μg), nalidixic acid (Nal; Sigma; 30 μg), metronidazole (Met; Sigma; 5 μg), 2,3,5 tetrazolium chloride (TTC; Sigma; 600 μg), 5-fluorouracil (5FU; Sigma; 60 μg), and sodium arsenite (NaAr; BDH; 20 μg) were added and the plates were incubated microaerobically at 37 °C for 24 h. Zones of inhibition > 10 mm in diameter were recorded as sensitive, and the results were converted into a two-digit resistotype. The first digit represents the sum of the scores for the first three agents, Tet, Nal and Met scoring 1, 2 or 4 for resistance respectively, and the second represents the score for the remaining agents, TTC, 5FU and NaAr, scoring 1, 2 or 4 respectively (e.g. a susceptibility pattern of SSRSRS converts to resistotype 42). Disks were prepared by adding 25 μl of an aqueous solution of the agent at an appropriate concentration to each disk, freezing at −20 °C for *c.* 1 h, and freeze drying; disks were stored at −20 °C.

Biotyping

Isolates were typed in the Lior [4] species and biotype scheme, using the testing methods for hippurate hydrolysis, H_2S production and DNAase activity described by Bolton *et al.* [24].

Pyrolysis mass spectrometry

The blind coded isolates of the initial trial comprised: 6 from 3 local family outbreaks; 29 thought to represent sporadic infections in the Cardiff area; 12 and 13 isolates respectively from two larger outbreaks contributed from a collection at Manchester PHL; and 16 replicate cultures taken at random from this

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isolate set. The later batch comprised those isolates from the clusters and outbreak detected during the resistotyping trial and a further assortment of 12 isolates throught to represent sporadic infections. These were grown at 42 °C for 48 h microaerobically on blood agar plates poured from the same batch of medium. For each culture, four pyrolysis foils were smeared with colony material, heated to 80 °C for 10 min within 10 min of sampling, to destroy enzyme activity and dehydrate the specimen, and stored in a vacuum desiccator for analysis. The prepared foils were processed in a PYMS 200X automated pyrolysis mass spectrometer, pyrolysing for 4 s at 530 °C [21].

The mass spectra were normalized to correct for variation in sample size, and univariate statistics were calculated for each mass. Masses showing within-culture coefficients of variation $< 7{\cdot}5\%$ were submitted to discriminant analysis, a multivariate statistical strategy which compares spectra and extracts significant inter-culture pattern differences as complex derived axes, termed canonical discriminant functions. The sum of the squares of the differences on these axes approximates to a χ^2 estimate of significance of difference on (number of cultures − 1) degrees of freedom. Culture-mean coordinates on these axes were submitted to cluster analysis in the Clustan program suite, with squared Euclidian distance as the dissimilarity measure and UPGMA hierarchical clustering, yielding a dendrogram and similarity matrix [21–23].

RESULTS

Applicability of resistotyping and biotyping

Within- and between-batch reproducibility of the biotyping and resistotyping tests was assessed for 14 analyses of 2 control strains, duplicate analyses of another 37 isolates and 5 repeat isolates from 5 patients. No disagreements were found, indicating a reproducibility of $> 98\%$. Interpretation of zone sizes was straightforward, with occasional exceptions for 5FU where secondary growth can produce a light swarm into a zone of inhibition. Costs were low; *c.* 53p in materials and 6 min processing time per isolate for resistotyping, and an additional *c.* 10p in materials and 5 min processing time per isolate for biotyping (assumes batch processing). The methods were suited to typing of single isolates and results could be available within 24 h (resistotyping) or 48 h (biotyping) of obtaining a pure culture.

Table 1. *Variation of isolation rates during the trial*

Month	Number of isolates
January	28
February	22
March	27
April	22
May	49
June	46
July	40
August	50
September	44
Total	328

Application of resistotyping and biotyping

During the first 9 months of the trial, 328 new cases of campylobacter enteric infection were confirmed by culture, excluding epidemiologically linked cases. The seasonal distribution (Table 1) was typical of previous years, with a clear peak in late spring and high incidence in the summer months. Resistotyping showed a broad distribution of patterns, with 35 distinct types found out of a possible 64, and biotyping showed a predominance of *C. jejuni* (85·9% of all isolates), particularly types I and II (Table 2). Discrimination was good, with no single resistotype accounting for $> 25\%$ of the isolates. The most frequently encountered resistotypes were, in descending frequency order, 40, 02, 00, 42 and 44. The probability that two independent isolates might fortuitously yield the same resistotype varied between 0·05 and 10^{-5}, depending upon the resistance pattern. When resistotyping and biotyping results were combined (Table 2) a total of 86 types was seen out of a possible 512, with no single group accounted for $>$ 10% of the isolates.

Infected patients with a recent history of foreign travel tended to yield isolates with unusual resistotypes, and a significantly ($P < 0{\cdot}0001$) higher proportion of *C. coli* isolates (Table 3). There were six incidents where there was temporal and geographical clustering, five of which involved only household contacts. Each of the latter five incidents yielded isolates of a single resistotype and biotype that formed clear tight clusters in PMS (Table 4).

The remaining incident involved 14 children and 1 adult from a local school who had drunk unpasteurized milk during a visit to a farm, together with 3 secondary cases. Briefly, all 19 isolates tested from these 18 patients were of resistotype 02; 17 (16

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Table 2. *Frequency of resistotypes and biotypes, excluding known repeat isolates and epidemiologically linked isolates of the same bio/resistotype*

	Frequency (%) of biotype							
	C. jejuni				*C. coli*		Frequency (%) of resistotype	Total number of isolates
Resistotype	I	II	III	IV	I	II		
00	1·5	4·9	2·1	—	1·5	0·6	10·7	35
01	0·6	—	—	0·3	—	—	0·9	3
02	7·9	2·7	—	—	0·3	0·3	11·3	37
03	—	0·3	—	—	—	0·6	0·9	3
04	0·6	—	0·3	—	0·6	—	1·5	5
05	—	—	0·3	—	—	—	0·3	1
06	0·3	—	—	—	—	—	0·3	1
10	0·3	0·3	—	—	—	—	0·6	2
20	—	—	—	—	—	0·3	0·3	1
30	—	—	—	—	—	0·3	0·3	1
40	7·9	9·5	1·5	0·9	0·9	1·2	22·0	72
41	1·2	0·3	0·3	—	0·9	0·3	3·0	10
42	7·3	2·7	—	—	0·3	—	10·4	34
43	0·6	0·3	—	—	—	0·6	1·5	5
44	2·1	2·7	3·0	1·5	0·3	0·6	10·4	34
45	2·7	0·3	0·3	—	—	0·3	3·6	12
46	1·5	0·6	—	—	—	—	2·1	7
47	0·9	0·3	—	—	—	—	1·2	4
50	3·0	1·2	—	—	0·3	—	4·6	15
51	0·6	1·2	—	—	0·9	—	2·7	9
52	1·2	0·3	—	—	—	0·3	1·8	6
53	0·6	—	—	—	0·3	0·3	1·2	4
54	0·6	—	—	0·3	0·6	—	1·5	5
55	—	0·3	—	—	—	—	0·3	1
56	0·6	0·3	0·3	—	0·3	0·3	1·8	6
57	0·3	—	—	—	—	—	0·3	1
60	—	—	—	—	0·3	—	0·3	1
61	0·3	—	—	—	—	—	0·3	1
62	0·9	—	—	—	—	—	0·9	3
64	0·3	—	—	—	—	—	0·3	1
66	—	0·3	—	—	—	—	0·3	1
70	—	0·3	—	—	—	—	0·3	1
71	0·6	0·3	—	—	—	—	0·9	3
72	—	0·3	—	—	—	—	0·3	1
73	—	0·3	—	—	—	—	0·3	1
Frequence of biotype (%)	44·8	29·9	8·2	3·0	7·6	6·1	100·0	—
Total number of isolates	147	98	27	10	25	20	—	327*

* One further strain of *C. lari* biotype II, resistotype 70, was also isolated.

patients) were biotype *C. jejuni* I and 2 were biotype *C. jejuni* II. Fifteen isolates were typed in PMS, and formed a clear cluster, with the exception of one biotype *C. jejuni* I which was distinct. All 19 isolates were also analysed in phage typing, serotyping, ribotyping and pulse field gel electrophoresis (Dr R. J. Owen, personal communication). The isolates formed a clear cluster in all these analyses, with three exceptions: the two *C. jejuni* biotype II isolates and the *C. jejuni* biotype I isolate that was distinct in PMS.

Two further pairs of isolates were also linked and yielded isolates of identical restisto- and bio-types. One involved two international rugby players who became ill within a day of one another while returning

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Table 3. *Association of biotype distribution with history of recent foreign travel*

Travel history	Number (%) of isolates of *C. jejuni* I+II	*C. coli* I+II	Total number of isolates
Foreign	22* (65)	12* (35)	34
UK or none	223 (90)	25 (10)	248
Total	245 (87)	37 (13)	282

* Includes several resistotypes not encountered among the UK isolates.

from a tour (Table 4), and one involved isolates from a patient, and from a sample of chicken that he had consumed (resistotype 44, *C. jejuni* biotype IV).

Pyrolysis mass spectrometry

In the initial trial, clusters corresponding to the replicate cultures, family outbreaks and the two larger outbreaks were readily identified and all the sporadic isolates were distinct, with the exception of one indistinguishable pair. There was great diversity of overall cell composition between distinct campylobacter strains, but also unusually wide variation between duplicate cultures and between repeat isolates. These tendencies counterbalanced to give a level of discrimination comparable to PMS typing studies of other species. Similar results were found for the resistotyping trial isolates; all sporadic isolates were distinct and clustering agreed with epidemiological and resistotyping data for the remaining isolates, with the exception of a single isolate from a farm outbreak described above.

DISCUSSION

The resistotyping method was highly reproducible within our laboratory and gave a level of discrimination better than that for O and H serotyping of salmonellae. For the 176 salmonellae (excluding repeat isolates from outbreaks and individual patients) isolated during the concurrent 9 months, there were 15 serotypes, with 1 accounting for 64% of isolates, compared to 35 resistotypes of campylobacter with none accounting for > 25% of isolates. This comparison is clearly biased by differences in epidemiology between the salmonellae and campylobacters. Nonetheless, the performance of salmonella typing provides the convenient yardstick of a method widely acknowledged as possessing sufficient discrimination for routine national surveillance.

Lior biotyping, although slower, in theory could increase the number of distinct campylobacter types eightfold. For this survey, biotyping increased the number of detected types to 86, and decreased the maximum proportion of isolates in any individual type twofold. The costs for this additional discrimination were small, but a further delay of 24 h was involved. By contrast, further discrimination of the concurrently isolated salmonellae by phage typing at the reference laboratory increased the number of distinct groups to 40 and involved significant delay. The two predominant salmonella types detected (*S. enteritidis* PT4 and *S. typhimurium* PT104) comprised 45% and 13% of isolates compared to the three predominant campylobacter bio-resistotypes (*C. jejuni* biotype II resistotype 40, *C. jejuni* biotype I resistotype 40, and *C. jejuni* biotype I resistotype 02) which constituted 9·5%, 7·9% and 7·9% of all campylobacter isolates, respectively. We suggest that a convenient abbreviated nomenclature might be a short-form Lior biotype followed by an R and the two digit resistotype, e.g., CjIIR40 would indicate a *C. jejuni* biotype II, resistotype 40 isolate.

Resistotyping methods that include commonly-used antibiotics such as tetracycline and nalidixic acid may be affected by rising resistance levels due to selection pressure. We believe that isolates yielding consistently distinct antibiograms should be considered distinct strains. Such changes in the resistance pattern must reflect genetic changes in the chromosome or plasmid DNA, and so an isolate that has acquired resistance is genetically distinct from the sensitive ancestor clone. Conversely, we make no claim that a resistotype represents a clone. Clearly, the nature and arrangement of genes coding for a specific resistotype may vary; as with all typing methods, resistotyping can indicate that isolates are distinct, but cannot unequivocally confirm that isolates are identical.

Another aspect of increasing resistance is degradation of resistotype discrimination. This may be less significant in thermophilic campylobacters, where our knowledge of epidemiology indicates that the main resevoirs are birds, farm and wild animals and pets, and long chains of person to person transmission are uncommon. Resistance levels to tetracycline, nalidixic acid and metronidazole in this survey were 17%, 3% and 58% respectively. For independent bi-state tests, discrimination reaches a maximum when

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Table 4. *Typing results for isolates from the clusters and linked isolates detected*

Cluster	Outbreak type	Number of patients	Biotype	Resistotype
A	Household	2	CjII	02
B	Household	2	CjI	42
C	Household	3	CjI	42
D	Household	2	CjIII	00
E	Household	2	CjIV	54
F	Milk-borne?	15	CjI	02
		1*	CjI	02
		2	CjII	02
G	Rugby tour	2	CjI	51

* Results in PMS and other methods indicated that this isolate was distinct.

the frequency of positive results for each test is 50%. Therefore, increased resistance to tetracycline or nalidixic acid would probably increase discrimination of resistotyping.

The level of discrimination achieved in resistotyping was impressive, and clearly adequate for routine local surveillance of campylobacters. The bio/resistotyping results for the six outbreaks were largely confirmed in PMS, and in the farm outbreak, by a broad range of methods (Dr R. J. Owen, personal communication).

Although typing was carried out retrospectively on batches of isolates during this survey, the method could readily cope with immediate typing of isolates. The presumptive milk-borne outbreak described coincided with the typing of a batch of isolates and the early results were a definite asset in tracing the outbreak. The impetus produced by early confirmation from typing encouraged a speedy, tightly directed investigation, while memory of the events was still clear in the minds of those involved in the outbreak. We propose to progress to immediate typing of individual isolates, or a much increased frequency of batchwise analysis.

This resistotyping method developed at Preston PHL is most promising. It is inexpensive, rapid, simple, highly discriminatory and shows excellent inter-batch stability. Further, it is well suited to use in local laboratories, fulfilling the need for rapid, local typing in outbreaks. With recent modifications to enhance inter-laboratory reproducibility (Dr F. J. Bolton, personal communication), it is a strong candidate for first-line use at a local level and for national surveillance of campylobacter infections. It would be an excellent tool for investigating suspected epidemiological associations such as that between cases and contamination of milk by magpies and other birds [25, 26].

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