Multilocus enzyme electrophoresis typing of clinical campylobacters from outbreak and sporadic sources

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Campylobacter jejuni is the most common cause of acute bacterial enteritis in Northern Ireland, as well as in the rest of the UK. Most recent data for 2000 record 1001 laboratory reports for Northern Ireland,[1] approximating to 59 cases per 100,000 individuals, compared to an attack rate of 106 and 127 cases per 100,000 individuals for England & Wales and Scotland, respectively. However, in a recent epidemiological study,[2] it was estimated that the true prevalence of this infection is approximately 10.3-fold higher, due to patient under-reporting.

Campylobacter spp. have a natural reservoir in the intestines of a wide range of feral and domesticated animal and birds, and enter the human food chain on raw animal products such as poultry, red meats and offal.[3] Untreated milk has been the vehicle for several large outbreaks and campylobacters can be found in inland and coastal waters as a result of faecal contamination by animals and sewage discharge.

Human infections caused by *Campylobacter* spp. arise from direct contact with animals or through contact with naturally contaminated raw or undercooked food products. Fortunately, large outbreaks of disease are rare and the majority of infections are considered sporadic. However, the vehicle for infection in most cases remains unidentified.

The epidemiology of *C. jejuni* enteritis is hampered by the lack of a standardised identification and typing scheme. Few laboratories in the UK identify their isolates to species level; fewer still utilise any of the recognised typing schemes. The combined consequences are that there is scanty information about the frequency and distribution of strain types that cause human infection, and where they are to be found in the food chain.

Multilocus enzyme electrophoresis (MLEE) has been used extensively for many years in eukaryotic cell genetics and has become a standard method in evolutionary biology.[4-6] In MLEE, allelic variation in a structural gene is detected by assessing the net electrostatic charge of the polypeptide encoded. The rate of migration of a protein during electrophoresis is determined by the amino acid sequence of the peptide (i.e., the net electrostatic charge). Hence, the mobility variants (electromorphs or allozymes) of an enzyme can be equated directly with alleles at the corresponding structural gene locus.

As different structural genes are represented by different enzymes, the choice of gene product allows the visualisation of specific bands after electrophoresis of cell lysates. Consequently, data provided by this method not only allow consistent identification of strains for epidemiological purposes but also a measurement of genetic distances among strains.

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Previously, there have been relatively few reports on the use of MLEE to differentiate between outbreak and sporadic isolates of *Campylobacter* spp., particularly *C. jejuni*.[7-8] This study aims to evaluate the ability of MLEE to discriminate between unrelated isolates with a panel of strains representative of common sporadic isolates from cases of human enteritis in the UK, as well as to examine the ability of MLEE to cluster outbreak strains.

Sets of strains from well-characterised outbreaks are incorporated to determine the reproducibility of these methods for identifying outbreak clusters. In addition, the study will evaluate the technique in terms of practicability and cost, repeatability, reproducibility, typeability, degree of discrimination and potential application in NHS clinical microbiology laboratories.

Seventy-six *C. jejuni* isolates previously characterised by Penner serology[9] Preston phagetyping[10] and Preston biotyping[11] (resistotype) were examined. A list of the strains used in the study and their phenotypic characteristics is shown in Table 1. This collection consisted of 36 isolates from nine well-characterised outbreaks of human campylobacteriosis and 40 sporadic isolates with no known epidemiological association.

Reference strains for the heat-stable serotyping scheme of Penner *et al*.[9] were obtained from Dr. D. M. Jones, Manchester Public Health Laboratory. Representative clinical laboratory isolates and isolates from outbreaks A, B, C, H and J were selected from the culture collections at Preston Public Health Laboratory and Manchester Public Health Laboratory. Outbreak D isolates were received from Dr. R. J. Owen, Central Public Health Laboratory, Colindale, London, who had obtained them from Professor H. Goossens (WHO Collaborating Centre for Enteric Campylobacter, St. Pierre University, Brussels, Belgium). Isolates from outbreaks E, F and G were kindly donated by Dr Bala Swaminathan, Centers for Disease Control (CDC), Atlanta, USA.

C. jejuni strains were grown on Campylobacter blood-free agar for 24 h at 37°C in a microaerobic atmosphere. The growth from one agar plate was harvested with a cotton-tipped swab and suspended in 1 mL 20% (w/v) glycerol in brain-heart infusion broth (Difco Laboratories, Detroit, USA) in 1.5 mL screw-capped plastic vials (Alpha Laboratories, UK) and stored at –70°C.

Cultures were taken from storage at –70°C and allowed to thaw at room temperature. A cotton-tipped swab was charged from the vial and used to inoculate a Campylobacter blood-free agar plate, which was spread for discrete colonies and incubated at 37°C for 72 h in a microaerobic atmosphere. Subsequent culture of all strains was on Campylobacter blood-free agar, and plates were incubated at 37°C for 24 h in a microaerobic atmosphere. Isolates were randomised and blinded before forwarding for MLEE analysis.

Isolates were cultured on Columbia agar (CM331, Oxoid, UK) supplemented with 5% (v/v) horse blood and incubated in 5% O_2/10% CO_2/85% (v/v) N_2 for seven days. For each isolate, 16 plates were used to increase cell biomass for enzymic extraction. Cells (approximately 10^{11} colony-forming units [cfu]) were obtained by cultivating *C. jejuni* as described above. They were then suspended in 2 mL chilled

Table 1. Origins of clinical outbreak and sporadic *C. jejuni* isolates employed in the study.

Isolate reference	Penner serotype	Preston phagetype	Preston biotype	Source
AI	4/13/50	CC28	6000	Milk outbreak (Glastonbury)
A2	4/13/50	CC28	6000	Milk outbreak (Glastonbury)*
A3	4/13/50	CC28	6000	Milk outbreak (Glastonbury)*
A4	2	CEA9	6004	Sporadic human isolate (Edinburgh)
AS	2	CEA9	6000	Sporadic human isolate (Bath)
A6	4/13/50	CC28	6000	Milk outbreak (Glastonbury)
A7	4/13/50	CC28	6000	Milk outbreak (Glastonbury)
A8	4/13/50	CC28	6000	Milk outbreak (Glastonbury)
A9	17	CE29	6020	Milk outbreak (Kettering)
A10	17	CE29	6020	Milk outbreak (Kettering)
AI1	1	0010	6014	Sporadic human isolate (Gateshead)
A12	38	FFF	6112	Serotype reference strain
A13	33	3146	6302	Serotype reference strain
A14	32	0042	6002	Serotype reference strain
AI5	55	NT	6102	Serotype reference strain
A16	10	CC88	6410	Serotype reference strain
A17	9	NT	6112	Serotype reference strain
A18	43	NT	6006	Serotype reference strain
A19	41	IC98	6012	Serotype reference strain
A20	53	NT	6016	Serotype reference strain
A2I	50	NT	6020	Serotype reference strain
A22	42	1010	6102	Serotype reference strain
A23	11	2142	6014	Sporadic human isolate (Norwich)
A24	4/50	CC28	6000	Sporadic human isolate
A25	4/13/50	CC28	6000	Milk outbreak (Glastonbury)*
A26	17	CFCB	6014	Institutional outbreak (Nottingham)
A27	17	CFCB	6014	Institutional outbreak (Nottingham)
A28	3	1000	6000	Serotype reference strain
A29	45	0004	6062	Serotype reference strain
A30	44	CC08	6010	Serotype reference strain
A31	1	CE29	6000	Sporadic human isolate (Edinburgh)
A32	1	2FEF	6124	Sporadic human isolate
A33	17	0001	6124	Serotype reference strain
A34	13/50	CC38	6000	Sporadic human isolate
A35	11	2142	6134	Serotype reference strain
A36	6	0142	6102	Serotype reference strain
A37	17	CE29	6020	Kettering milk outbreak
A38	40	1000	6012	Serotype reference strain
A39	17	CFCB	6014	Institutional outbreak (Nottingham)

(2°C) buffer (10 mmol/L Tris, 1 mmol/L EDTA, 0.5 mmol/L NADP [pH 6.8]).

Lysates were prepared by exposing the suspended cells to two cycles of sonication (30 sec) in an MSE 150-W Mk 2 ultrasonic disintegrator (Sanyo, Tokyo, Japan) on minimal power and cooled in an ice bath. The cells and particles remaining after lysis were removed by centrifugation (30,000 x*g* for 15 sec at 4°C) and the straw-coloured lysates were stored in 200 µL amounts at –70°C.

All the isolates were assayed for the following nine enzymes: malate dehydrogenase (MDH; E.C. 1.1.1.37), isocitrate dehydrogenase (IDH; E.C. 1.1.1.42), indophenol oxidase (IPO; E.C. 1.15.1.1.) (superoxide dismutase), malic enzyme, alkaline phosphatase, fumarase, adenylate kinase (ADK; E.C. 2.7.4.3), peptidase of L-phenylalanyl-L-leucine (PEP) and aconitase (ACO; E.C. 4.2.1.3).

The generic MLEE method, described previously by Selander *et al.*,[12] was employed for electrophoresis and staining. Visual comparisons of the mobilities of the enzymes from the different isolates were made on the same gel slice with the aid of an illuminated lightbox. Replicate control strains were analysed on each gel slice, which

Table 1 (continued). Origins of clinical outbreak and sporadic *C. jejuni* isolates employed in the study.

Isolate reference	Penner serotype	Preston phagetype	Preston biotype	Source
A40	17	CFCB	6014	Institutional outbreak (Nottingham)
A41	5	NT	6400	Serotype reference strain
A42	4	NT	6110	Serotype reference strain
A43	13/16/50	DC38	6000	Sporadic human isolate (Bath)
A44	13/16/50	0A98	6114	Sporadic human isolate (Norwich)
A45	1	3FFF	6010	Poultry isolate (Scotland)
A46	1	DEB9	6000	River water isolate (Aberdeen)
A47	2	1010	6014	Sporadic human isolate (Rhyll)
B1	18	2146	6000	Meningitis outbreak (France)
B2	18	2146	6000	Meningitis outbreak (France)
B3	18	2146	6000	Meningitis outbreak (France)
B4	18	2146	6000	Meningitis outbreak (France)
B5	18	2146	6000	Meningitis outbreak (France)
B6	18	2146	6000	Meningitis outbreak (France)
B7	18	2146	6000	Meningitis outbreak (France)
B8	18	2146	6000	Meningitis outbreak (France)
B9	18	2146	6000	Meningitis outbreak (France)
B10	18	2146	6000	Meningitis outbreak (France)
B11	2,1	DEB9	6000	Milk outbreak (Vermont, USA)
B12	22, 23, 36	NT	6002	Milk outbreak, (Kansas, USA)
B13	22, 23, 36	NT	6002	Milk outbreak, (Kansas, USA)
B14	19	2146	6004	Water outbreak (Florida, USA)
B15	19	2146	6004	Water outbreak (Florida, USA)
B16	2	DEB9	6000	Milk outbreak (Vermont, USA)
B17	22, 23, 36	NT	6002	Milk outbreak, (Kansas, USA)
B18	22, 23, 36	NT	6002	Milk outbreak, (Kansas, USA)
B19	19	2146	6004	Water outbreak (Florida, USA)
B20	2	1010	6004	Pate outbreak A (Stoke)
B21	2	1010	6004	Pate outbreak A (Stoke)
B22	2	1010	6004	Pate outbreak A (Stoke)
B23	11	2146	6010	Pate outbreak B (Stoke)
B24	11	2146	6010	Pate outbreak B (Stoke)
B25	18	2146	6000	Meningitis outbreak (France)
B26	22, 23, 36	NT	6002	Milk outbreak, (Kansas, USA)
B27	19	2146	6004	Water outbreak (Florida, USA)
B28	2	DEB9	6000	Milk outbreak (Vermont, USA)
B29	11	2146	6010	Pate outbreak B (Stoke)
B30	2	1010	6004	Pate outbreak A (Stoke)

* Isolate was linked with the Glastonbury milk outbreak but was unconfirmed

facilitated easy comparison between the gels. For each enzyme, distinctive electromorphs were numbered in order of decreasing anodal migration.

The repeated absence of enzymic activity was scored as a null character (0). Each isolate was characterised by its combination of electromorphs over the nine enzymic loci assayed and distinct profiles of electromorphs, corresponding to unique multilocus genotypes were designated electrophoretic types (ETs).

MLEE typing was applied to 76 isolates and typing results for outbreak-related isolates and sporadic isolates are shown in Tables 2 and 3, respectively. Isolates from two of the nine outbreak clusters were grouped correctly into indistinguishable clusters. In five of the outbreak sets tested where discordant results were found, only one aberrant result was found within each cluster.

Isolates from outbreak H were divided into two profiles (MLEE subtypes 12 and 13). The three isolates in outbreak J were each found to have a unique MLEE profile (i.e., MLEE types 14, 15 and 16). In five outbreak sets (D, E, G, H and J), one strain was replicated and tested. MLEE results for the replicate strains were concordant in three of the pairs.

Table 2. Multilocus enzyme electrophoresis (MLEE) analysis of outbreak isolates

Isolate reference (replicate of)	Outbreak code	MLEE subtype
AI	A	1
A2	A	1
A3	A	1
A6	A	1
A7	A	1
A8	A	1
A25	A	1
A9	B	2
A10	B	2
A37	B	2
A26	C	3
A27	C	3
A39	C	4
A40	C	3
B1	D	5
B2	D	5
B3	D	5
B4	D	5
B5	D	5
B6	D	5
B7	D	6
B8	D	5
B9	D	5
B10	D	5
B25(B1)	D	5
B11	E	7
B16	E	8
B28 (B11)	E	7
B14	F	9
B15	F	9
B27 (B19)	F	10
B12	G	11
B13	G	12
B17	G	11
B18	G	11
B26 (B13)	G	11
B20	H	12
B21	H	13
B22	H	13
B30 (B21)	H	12
B23	J	14
B24	J	15
B29 (B24)	J	16

Twenty-seven distinct MLEE profiles were found among the 33 sporadic and reference strains tested.

In this study, MLEE was selected as a differentiating scheme because it is a technique that does not require DNA sequencing or alternative techniques in order to analyse the genetic structure of clinical *C. jejuni* isolates. The technique's main advantage is that variation in mobility of a cellular enzyme can be related directly to allelic variation in specific genes encoding specific proteins. In addition, Hartl and Dykhuizen[13] demonstrated that much of the polymorphic variation in enzymes is selectively neutral and so is only minimally subject to evolutionary convergence.

In terms of repeatability, several discrepancies were detected where replicate isolates failed to produce an identical profile. The reproducibility of the method was moderate. The main reproducibility problem encountered was inter-gel variation. Unlike other genotypic typing methods, MLEE profiles comprise results of a composite of separate gels, and inter-gel variation can be a significant factor.

Similar strains were rechecked by running additional gels to compare similar ET profiles and it was concluded that computer-based recording and storage of gel data would eliminate the need for this rechecking. Detailed analysis of the discrepancies showed that the discordant results were due to inter-gel variation and should have been detected by comparing the stored images.

One discordant result was probably due to contamination and the other was unexplained. Improved use of internal controls equivalent to a 'molecular weight' ladder in each gel may improve inter-gel comparisons but further work is needed in this area.

An alternative method of manipulating the results to remove error is to use the 'clonal cluster' concept. If electromorph types are designated by genetic distance then the level at which types are discriminated could be set to eliminate minor differences caused by technical or data interpretation problems. This would decrease the discrimination of the technique but might improve the reliability of the method. However, the threshold genetic distance chosen to discriminate significantly different strains would have to be determined using a larger database of characterised strains.

In terms of typeability, all the isolates produced an MLEE subtype profile. This technique detects the electrophoretic mobility of constitutively produced enzymes and consequently a result is always generated for each of the enzymes examined. Hence, it is unlikely that any campylobacters would be non-typeable by MLEE analysis.

This technique proved highly discriminatory with the sporadic strains tested. Very few MLEE profiles were seen in more than one isolate. However, as discussed above, the level of discrimination is relative to the measure of a true difference between strains and it may be possible to establish different levels of discrimination for individual purposes by determining different thresholds to discriminate major and minor differences. For example, more detailed analysis may be required to investigating outbreak clusters than is needed for general population studies or for surveillance purposes.

MLEE is relatively easy to perform, although it is labour-intensive and throughput is slow – approximately 16 strains can be tested in each batch. Results may take up to four days to be produced. Preparation of good-quality gels and evaluation of data to produce MLEE profiles requires an experienced operator to generate reliable results.

The technique has low capital and moderate running costs. However, the equipment required is unlikely to be available in most routine laboratories and would limit the applicability

Table 3. Multilocus enzyme electrophoresis (MLEE) analysis of sporadic isolates

Isolate reference	Isolate type	MLEE subtype
A11	Sporadic	G
A46	Sporadic	E
A45	Sporadic	E
Al6	Reference	B
A23	Sporadic	J
A35	Reference	H
A4	Sporadic	L
A5	Sporadic	M
A47	Sporadic	N
A36	Reference	Q
A28	Reference	O
A14	Reference	S
A13	Reference	T
A12	Reference	U
A38	Reference	V
A19	Reference	W
A22	Reference	X
A30	Reference	Z
A29	Reference	A'
A18	Reference	Y
A21	Reference	A
A24	Sporadic	A
A34	Sporadic	A
A42	Reference	I
A43	Sporadic	A
A44	Sporadic	K
A41	Reference	P
A20	Reference	B'
A15	Reference	C'
A31	Sporadic	B
A32	Sporadic	D
A33	Reference	D
Al7	Reference	R

of the technique to specialist centres, particularly if image capture hardware and software are required.

Nonetheless, the method provides a valuable typing tool, especially to examine the population genetics and clonal evolution of bacterial species, as demonstrated with taxa such as *Neisseria meningitidis,*[14] *Pasturella*[15] and *Corynebacterium*[16] spp. Thus, any modification to, or simplification of, the existing laboratory parameters should be welcomed, to encourage greater willingness to employ this technique in the examination of epidemiological relationships and the population genetics of *C. jejuni*.

More recently, multilocus sequence typing (MLST) has emerged as an important typing tool in bacterial population genetic studies.[17, 18] This has now been applied successfully to the subtyping of a wide variety of bacterial and fungal pathogens including *Enterococcus faecium*,[19] *N. meningitidis*[20] and *Candida albicans*,[21] as it has proved more reproducible than MLEE.

Increased use of MLST is mainly a result of the widespread adoption and availability of automated DNA sequencing in microbiology laboratories, as well as improvements over existing typing techniques such as pulsed-field gel electrophoresis (PFGE) and MLEE. MLST examines nucleotide differences in several gene loci, whereas MLEE relies on cellular detection of variation at these gene loci. To date, there have been at least two reports of the use of MLST in the subspecies characterisation of *C. jejuni*, where it has shown that the population structure of *C. jejuni* is weakly clonal.[22,23]

In conclusion, MLEE is a valuable subspecies typing technique and an alternative to several molecular typing methods based on the examination of polymorphisms within hypervariable gene loci (e.g., *flaA*) for laboratories that do not possess molecular (PCR and sequencing) capability. However, given its relative complexity and demand for time, it is unlikely that this method will be adopted for routine typing in hospital clinical microbiology laboratories but will be reserved for research applications in specialist and reference laboratories. □

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Whole gene pneumolysin PCR can be used as a diagnostic assay but cannot predict serotype

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Streptococcus pneumoniae is responsible for infections such as pneumonia, bacteraemia and meningitis. Despite antimicrobial therapy, invasive pneumococcal disease (IPD) remains a leading cause of morbidity and mortality worldwide, especially in the young and old.[1-3]

S. pneumoniae produces a number of virulence factors, one of which is pneumolysin. This is a 53 kDa polypeptide comprising 470 amino acids, encoded by the pneumolysin (*ply*) gene. Pneumolysin interferes with phagocyte function *in vitro* by exerting haemolytic activity and suppressing immune function.[4] This multifunctional virulence factor is produced by almost all clinical *S. pneumoniae* isolates and could be a suitable candidate for use in the detection of invasive *S. pneumoniae* infection.

Isolates of *S. pneumoniae* are serotyped according to their capsular type and can be characterised using multilocus sequence typing (MLST). The sequence type (ST) is derived from the nucleotide sequencing of housekeeping genes, which are unrelated to capsular type and often show a correlation between serotype and sequence type (unpublished data).

Polymerase chain reaction (PCR) methods have been described for the non-culture confirmation of *S. pneumoniae*.[5-10] Sequence variation has also been reported within the *ply* gene of serotypes 7 and 8.[11] Hence, sequence variation within the *ply* gene may provide the basis of a new typing method for pneumococci, based on the relationship between sequence variation of the *ply* gene and serotype.

Here, the development and evaluation of a whole gene *ply* PCR for the confirmation of IPD from body fluids is described. Also sequenced are the *ply* genes from the 23 serotypes (included in the 23-valent polysaccharide vaccine) that cause 96% of IPD to evaluate their suitability for use as a sequence-based typing method for the identification of different serotypes.

Ten strains each from the 23 *S. pneumoniae* serotypes were taken from the collection held by the Scottish Meningococcus and Pneumococcus Reference Laboratory (SMPRL). Five hundred clinical samples (whole blood and cerebrospinal fluid [CSF]) previously tested for pneumococcal antigen also were used to evaluate the assay. Genomic DNA was extracted from blood samples using the Nucleospin blood kit (Abgene, Epsom, UK), as described previously.[12] CSF samples did not require extraction. DNA was extracted from pneumococcal isolates by adding 10 colonies to 200 µL distilled water. The suspension was heated to 80°C for 4 min, centrifuged for 3 min at 13000 x*g* and the supernatant frozen at –70°C for a minimum of 1 h and then thawed before addition of the PCR mix.

PCR primers were designed from the published pneumolysin gene sequence (Genbank accession number M17717; www.ncbi.n/m.nih.gov/BLAST/) using Gene Fisher (http:/bibiserv.techfak.uni-bielefeld.de/genefisher/) (Table 1). The PCR reaction mix contained 21 µL Reddymix (Abgene), 1 µL each primer (2 pmol stock) and 3 µL DNA. PCR cycling conditions used were as follows: 95°C for 2 min, 35 loops of 95°C for 1 min, 51°C for 1 min 30 sec and 72°C for 2 min, followed by 5 min at 72°C.

Owing to the low DNA concentration in the clinical samples, a nested PCR step was used with the initial PCR product, which was purified using the Millipore Multiscreen 384-PCR plate (Millipore, Watford, UK), as described previously.[13] Nested primers were designed and produced as above (Table 1). For the nested PCR, 20 pmol each primer was prepared and used in 1 µL volumes. The thermocycling conditions were as follows: 95°C for 2 min, 35 cycles of 95°C for 1 min, 50°C for 1 min 30 sec and 72°C for 2 min, finishing with 72°C for 5 min. The amplified nested product was then

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