examined for confluent lysis or plaques. Lysis was recorded as semi-confluent to confluent (+++), 50-100 plaques (++), 20-49 plaques (+) and 1-19 plaques (±). Only +++ or ++ phage reactions were recorded as positive. Phage patterns were recorded and assigned to one of eight previously designated phage groups, defined from different studies of more than 1000 sporadic isolates from different parts of the UK. Phage types with similar lytic patterns (i.e., two or less differences) were clustered to form groups.

The Preston biotyping scheme proved highly discriminatory and produced 20 biotypes for *C. jejuni* and eight biotypes for *C. coli* (Table 1). The phage-typing technique gave equal discrimination as 20 phage types were recorded for *C. jejuni* and three for *C. coli* (Table 2); however, it did produce a significant number of untypable isolates (20/117 [17.1%]). This may have been because the reference phages were too specific, possibly reflecting the fact that a limited pool of campylobacters were studied. Consequently, in order for phage typing to be considered an acceptable typing technique, additional phages need to be isolated from other sources in order to reduce this level of non-typability. Reference phages used in the scheme were isolated from a wide range of clinical and non-clinical sources.

Overall, this comparison of a limited number of Northern Ireland isolates with a larger group of isolates from England and Wales showed no marked difference in phenotype frequency, using either biotyping or phage typing as epidemiological markers. This would suggest that phenotypic differences do not account for the difference in attack rates between the groups and that Northern Ireland shares a similar pool of phenotypes with England and Wales.

Previously, LaFong and Bamford[6] suggested that differences in attack rates between Britain and Northern Ireland might be due to (a) relatively low consumption of unpasteurised milk in Northern Ireland, (b) a higher ratio of red meat to white meat consumption in Northern Ireland, (c) climatic factors and (d) a social likeness for 'well done' food in Northern Ireland.

The data presented here would support the hypothesis that such differences are probably due to social aspects of human behaviour (e.g., food preparation) rather than to any differences in isolate type.

We await the results of the current UK Campylobacter Sentinel Surveillance Scheme with interest, as this will permit a comprehensive and current comparison of Northern Ireland types with those from Britain and should help elucidate additional risk factors for human campylobacteriosis in Northern Ireland. □

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Multilocus enzyme electrophoresis typing of clinical *Helicobacter pylori*

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Multilocus enzyme electrophoresis (MLEE) has been used extensively for many years in eukaryotic cell genetics and has become a standard method in evolutionary biology.[1-3] Allelic variation in a structural gene can be detected using MLEE 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, choice of gene product permits the visualisation of specific bands following electrophoresis of cell lysates. Consequently, the data provided by this method not only allows consistent identification of strains for epidemiological purposes but also a measurement of genetic distances among strains.

Previously, only one study performed in the mid-1990s has used MLEE to type *Helicobacter pylori*.[4] Therefore, the present study aims to optimise laboratory parameters (including phase and basal growth medium) for the cultivation of *H. pylori* for MLEE analysis. In addition, optimised conditions are employed in a small preliminary study to examine genetic relatedness, based on cluster analysis of differences at nine enzyme loci in isolates associated with different gastric pathologies.

Details of the clinical isolates of *H. pylori* (n=12) used in the study are presented in Table 1. The identity of each was

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confirmed by demonstrating typical colony morphology, appearance on Gram stain and positive assays for urease, catalase and oxidase. Isolates were cultured on Columbia agar (Oxoid CM331; Oxoid, Basingstoke, UK) supplemented with 5% (v/v) horse blood and incubated (5% O_2, 10% CO_2, 85% (v/v) N_2) for seven days. For each isolate, 16 plates were employed to increase cell biomass for enzymic extraction.

Four commercially available non-selective basal broth media were examined in this study, including nutrient broth (NB; Oxoid CM1), tryptone soya broth (TSB; Oxoid CM129), brain heart infusion broth (BHI; Oxoid CM225) and Mueller-Hinton broth (MH; Oxoid CM405). All media were reconstituted following the manufacturer's instruction for preparation of the media. All strains were grown at 37°C for four days on Columbia Blood agar (Oxoid CM0331) supplemented with 5% (v/v) defibrinated horse blood under microaerophilic conditions (CampyPack Plus Gas Generating System; Oxoid BR56).

Isolates were harvested into 0.1% (w/v) peptone saline (Oxoid CM0009) to give an inoculum cell density equal to McFarland No. 2. Inocula (1 mL) were added to 100 mL of the four broth media containing 5 mL deactivated horse serum (Oxoid) in separate 200 mL tissue culture flasks (Falcon, UK). Culture flasks, with caps fitted loosely, were placed horizontally in a CO_2 incubator for 2 h. The caps were tightened and the flasks removed to a shaking incubator (Innova 4000, New Brunswick Scientific, UK) and incubated at 37°C at 176 rpm for 60 h.

Absorbance values (*A*) were recorded at zero, 24, 48 and 60 h by removing 250 µL samples of inoculated media into a 96-well, flat-bottomed microtitre plate (Sarstedt, Germany) without disturbing the microaerophilic atmosphere. For each treatment series, an equal number of negative controls were established, containing uninoculated media, to check sterility and thus avoid false-positives.

Plates were shaken and read spectrophotometrically (405 nm) on an automatic microtitre plate reader (Emax, Molecular Devices, Sunnyvale, CA, USA). *A* values were calculated by deducting the control value of the broth at time t from the value of the inoculated broth.

Cells (approximately 10^{11} colony forming units [cfu]) were obtained by cultivating *H. pylori* isolates in BHI broth for 48 h, as described above, and were harvested by centrifugation at 13,000 x**g** for 5 min in a refrigerated centrifuge (Haereus, Finland). Cells were resuspended in 2 mL of chilled (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 s) in an MSE 150-W ultrasonic disintegrator (Sanyo, Tokyo, Japan) on minimal power and cooled in an ice bath. Cells and particles remaining after lysis were removed by centrifugation (30,000 x**g** for 15 s at 4°C) and the straw-coloured lysates were stored in 200 µL amounts at –70°C.

All the isolates were assayed for 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 methods described previously by Selander *et al*.[5] were employed for electrophoresis and staining. Comparisons of the mobilities of the enzymes from

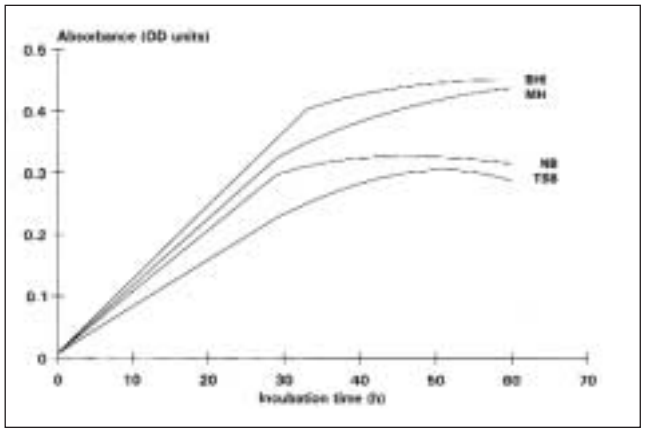

Fig. 1. Comparison of cell yield of 12 clinical isolates of *H. pylori* in four basal broth media.

the different isolates were made visually on the same gel slice with the aid of an illuminated light box. Replicate control strains were analysed on each gel slice, which facilitated easy visual comparison between the gels. For each enzyme, distinctive electromorphs were numbered in order of decreasing anodal migration. The repeated absence of an 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 distinctive profiles of electromorphs, corresponding to unique multilocus genotypes, were designated as electrophoretic types (ETs). Genetic diversity (h) was calculated for each locus as $(1-\Sigma x_i^2)[n/(n-1)]$, where x_i is the frequency of the ith allele at the locus, n is the number of ETs and [n/(n-1)] is a correction factor for the bias in small samples.[6] Pairwise comparisons between ETs were analysed statistically by calculating the Euclidean distance between clustered pairs of strains, which gave an unweighted matrix of coefficients of genetic distance over the enzymic loci.

Mean spectrophotometric values of cell density (corrected *A* units versus time) were plotted for each isolate for MH, BHI, NB and TSB (Figure 1). Mean *A* results of the broth media comparison for each strain allowed the basal media to be ranked (commencing with the medium that gave the greatest proliferation of organisms) into the order BHI>MH>NB>TSB. Isolate growth on Columbia blood agar was relatively poor and did not yield adequate quantities of cell biomass to produce sufficient cellular enzymes for subsequent electrophoresis and quantification.

Each *H. pylori* isolate examined in this study represented a distinct ET type, with no sharing of ET types between *H. pylori* derived from the same disease status (ET diversity = 1.00), and there was no association of MLEE type with gastric pathology. The mean number of alleles per locus was 3.778, with ACO scoring 3 (the lowest number) and PAL and ADK scoring up to 8 alleles per locus. The mean genetic diversity (i.e., the arithmetic average over all loci assayed), including monomorphic values, was 0.5910. A principal coordinate plot (Figure 2) was generated from the matrix of coefficients of genetic distance as a model for the genetic relationships among the nine loci and 12 isolates examined.

In this study, MLEE was selected as a differentiating scheme because it is a technique that does not require DNA sequencing, or alternatives, to analyse the genetic structure of clinical *H. pylori* isolates. Its 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[7] have demonstrated that much of the polymorphic variation in enzymes is selectively neutral and thus is minimally subject to evolutionary convergence.

MLEE is a labour-intensive laboratory method that requires relatively long analysis time and a diverse range of biochemical reagents. However, the method provides a valuable typing tool to examine the population genetics and clonal evolution of bacterial species, as demonstrated previously with several other taxa including *Campylobacter jejuni*,[8] *Neisseria meningitides*,[9] *Pasturella*[10] and *corynebacterium* spp.[11] Thus, any modification or simplification to the existing laboratory parameters should be welcomed. Of the broths examined in the present study, BHI proved the most efficient at biomass recovery, probably due to a higher proportion of peptone in the formulation of this medium (Table 2).

In the one previous description of MLEE typing of *H. pylori* in the literature,[4] Go *et al*. screened 24 enzyme loci and selected six (glucose-6-phosphate dehydrogenase, glutamate dehydrogenase, isocitrate dehydrogenase, indophenol oxidase, nucleoside phosphorylase and adenylate kinase) as suitable markers for diversity in *H. pylori*. In the present study we looked at seven loci (malate dehydrogenase, isocitrate dehydrogenase, indophenol oxidase, glyceraldehyde-phosphate dehydrogenase, adenylate kinase, peptidase of L-phenylalanyl-L-leucine and aconitase). Although the overall conclusions from each study were similar and showed no association between MLEE type and gastric pathology, we did note several differences.

Firstly, we did not observe such a high number of allelic types per locus. As the methods employed were similar, such differences may be due to the fact that Go et al. studied

Table 1. Origins of clinical *H. pylori* isolates employed in this study

Isolate No.	Date of isolation	Sex	Age	Clinical symptoms	Clinical diagnosis	Histological findings
AHB36	20-05-1996	M	33	epigastric pain	duodenitis	gastritis
FAB33	02-05-1996	M	62	epigastric pain	gastritis	gastritis
AHB57	13-08-1996	M	39	epigastric pain, nausea	duodenal ulcer	gastritis
AHB53	29-07-1996	M	32	epigastric pain, nausea, heartburn	duodenitis	gastritis
AHB50	09-07-1996	M	34	epigastric pain, nausea, heartburn	duodenitis	gastritis
P100	23-06-1992	F	58	epigastric pain	gastritis	gastritis
FAB68	18-06-1996	F	33	epigastric pain	duodenal scar	gastritis
FAB49	08-07-1996	M	29	epigastric pain, heartburn	normal	gastritis
FAB71	20-06-1996	F	50	heartburn	Barrett's oesophagitis	gastritis
FAB58	10-06-1996	M	69	epigastric pain	normal	gastritis
FAB54	20-05-1996	F	40	heartburn	oesophagitis	gastritis
FAB69	18-06-1996	F	43	heartburn	normal	gastritis

Table 2. Comparison of constituents of basal broth culture media employed in this study

	Broth formulation			
Constituents [g/L]	NB	TSB	BHI	MH
Lab-Lemco powder	1.0			
Dehydrated infusion from beef				300.0
Pancreatic digest of casein		17.0		
Brain heart infusion solids			3.5	
Casein hydrolysate				17.5
Papaic digest of soybean meal		3.0		
Yeast extract	2.0			
Peptone	5.0		25.0	
Starch				1.5
Glucose		2.5	2.0	
Sodium chloride	5.0	5.0	5.0	
Disodium hydrogen phosphate			2.5	
Dibasic potassium phosphate		2.5		
	pH 7.4±0.2	pH 7.3±0.2	pH 7.4±0.2	pH 7.3±0.1

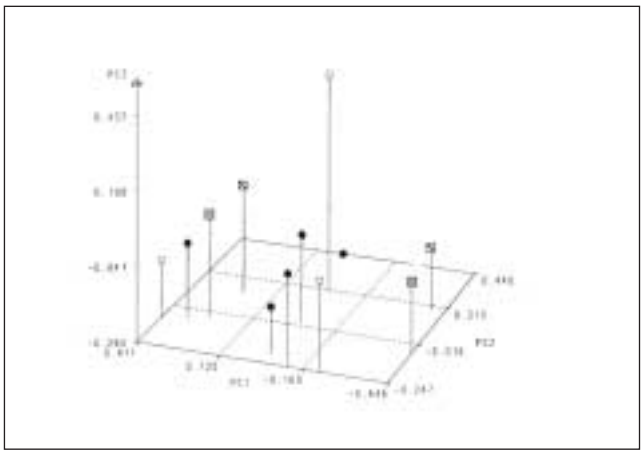

Fig. 2. A principal coordinate plot derived from a matrix of coefficients of genetic distance as a model for the genetic relationships between the 12 electrophoretic types of the 12 isolates examined. Key to symbols – ●: duodenitis; □: oesophagitis; ■: asymptomatic; O: gastritis.

clinical isolates from 74 individuals comprising African blacks, Hispanics, Caucasians and one Asian individual, whereas the present study looked at a smaller number of Caucasians only.

More recently, multilocus sequence typing (MLST) has emerged as an important typing tool in bacterial population genetic studies.[12] This technique has been applied successfully to the subtyping of a wide variety of bacterial and fungal pathogens including *Enterococcus faecium*,[13] *Neisseria meningitidis*[14] and *Candida albicans*.[15] The increased use of MLST is due mainly to the widespread adoption and availability of automated DNA sequencing equipment in microbiology laboratories. MLST examines nucleotide differences in several gene loci, whereas MLEE relies on cellular detection of variations at these gene loci.[16,17] To date, there have been no reports of the use of MLST in the subspecies characterisation of *H. pylori*, although the technique has been employed to examine clonality in campylobacter populations.[18]

In conclusion, MLEE provides a valuable subspecies typing technique and is an alternative to methods that examine polymorphisms within virulence loci (e.g., *vac*A, *cag*A, *ice*A) for laboratories that do not possess molecular (PCR + sequencing) capability. Our study concurs with others from non-related geographical areas, demonstrating the non-clonal nature of *H. pylori*.[4,19] It is important that optimised laboratory methods are used with this technique. Cluster analysis of 12 isolates associated with different gastric pathologies did not demonstrate any association between MLEE type and disease state, suggesting a high degree of heterogeneous gene rearrangements at the seven loci studied. Using the optimised parameters defined in this preliminary study, several hundred more isolates need to be examined to confirm the lack of association between MLEE type and disease state. □

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Extraction of genomic DNA from *Pseudomonas aeruginosa*: a comparison of three methods

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In diagnostic clinical microbiology there is growing interest in the adoption of molecular techniques to enhance and supplement conventional techniques. The majority of these molecular methodologies involve the use of extracted genomic DNA from bacterial cells for either diagnostic purposes and/or epidemiological subtyping. Regardless of the downstream application, all methods share a common procedure in the extraction of DNA from cells.

Previously our group has reported on the difficulties associated with the reliable extraction of bacterial DNA from blood cultures.[1] Here, we describe a simple observation that may be beneficial to laboratory personnel involved in the extraction of genomic DNA from bacterial pure cultures.

Pseudomonas aeruginosa isolates (n=60) were obtained by conventional selective culture from the sputa of adult cystic fibrosis (CF) patients attending the Northern Ireland Regional CF Centre. Three DNA extraction protocols were compared by several criteria as detailed in Table 1. For all methods, a loop (10 µL) of overnight culture of *P. aeruginosa* from Columbia blood agar (Oxoid CM0331; Oxoid , Bassingstoke, England), supplemented with 5% (v/v) defibrinated horse blood, was extracted.

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Method A (boiling): Bacteria were placed in molecular grade water (200 µL; BioWhittaker, Maryland, USA) and boiled for 10 min, then centrifuged at 12,000 x**g** for 5 min. The supernatant was stored at 4˚C.

Method B (QIAamp blood kit; Qiagen, Crawley, UK): Bacteria were placed in phosphate-buffered saline (PBS; 200 µL) and proteinase K (25 µL) was added along with 'binding buffer' (200 mL) as supplied. This was vortex-mixed immediately for 15 sec, or until the pellet dissolved. The mixture was incubated at 70°C for 10 min. DNA was extracted and eluted from spin columns, following the manufacturer's instructions, and then stored at 4°C.

Method C (High-purity PCR template preparation kit; Boehringer Mannheim, Lewes, UK): Bacteria were suspended in 200 µL of 10 mmol/L Tris-EDTA buffer (pH 8.0). Lysozyme (30 µL; pH 7.4) was added and the samples were incubated at 37°C for 45 min. Binding buffer (200 µL) and proteinase K (40 µL) were added and incubated at 72°C for 15 min. DNA was extracted and eluted from spin columns, following the manufacturer's instructions, and stored at 4°C.

Extracted DNA (15 µL) was examined by 2% (w/v) agarose gel electrophoresis (80V, 45min) and ethidium bromide (5 µg/100 mL) stained gels were visualised under ultraviolet illumination with a gel image analysis system (UVP Products, England). All images were archived as digital (*.bmp) graphic files. The ability to amplify extracted DNA was determined by a specific polymerase chain reaction (PCR) assay that targeted the outer membrane protein of *P. aeruginosa*, as previously described.[2]

The quality and quantity of extracted DNA obtained using the three methods are described in Table 1. All DNA extracts were able to generate a PCR amplicon of the correct molecular size.

The ability to extract genomic DNA from bacterial cells grown in culture is the cornerstone of any downstream molecular manipulation. Table 1 details the outcomes against several criteria for the three extraction methods employed; however, Wilson provides a comprehensive review of PCR inhibition and its causes.[3]

Overall, we would discourage use of DNA extraction from bacterial culture by boiling. We have experienced difficulty in the repeatability of downstream PCR reactions, particularly when boiling is used to extract DNA from pigmented isolates grown on agar culture and from cells that produce thermostable nucleases, including methicillin-resistant *Staphylococcus aureus* (MRSA),[4] and when such extracts are re-amplified after storage. Therefore, we would encourage the use of commercially available DNA extraction kits, which allow relatively rapid extraction of high-quality genomic DNA that is stable at 4˚C and can be used after

Table 1. Comparison of three methods for the extraction of genomic DNA from *Pseudomonas aeruginosa*

DNA extraction method	Speed	Quantity of DNA	Degree of shearing	Cost	Viability of DNA on storage at 4˚C
Boiling	Fast	Low	High	Low	Short (<7 days)
QIAamp blood kit (QIAGEN)	Moderate	High	Low	High	Long
High-purity PCR template preparation Kit (Boehringer Mannheim).	Moderate	High	Low	High	Long