Same-Day Subtyping of *Campylobacter jejuni* and *C. coli* Isolates by Use of Multiplex Ligation-Dependent Probe Amplification–Binary Typing

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Campylobacteriosis is the most commonly reported form of human bacterial gastroenteritis in the world. Sound identification of infectious sources requires subtyping, but the most widely used methods have turnaround times measured in days and require specialist equipment and skills. A multiplex ligation-dependent probe amplification-binary typing (MBiT) assay was developed for subtyping *Campylobacter jejuni* and *Campylobacter coli*. It was tested on 245 isolates, including recent isolates from Belgium and New Zealand, and compared to multilocus sequence typing (MLST). When used in an outbreak setting, MBiT identified the predominant genotype and possible additional cases days before pulsed-field gel electrophoresis (PFGE) results were available. MBiT was more discriminatory than MLST and, being a single assay with results produced within 6 h, was more rapid and cost-effective than both MLST and PFGE. In addition, MBiT requires only basic molecular biology equipment and skills.

*C*ampylobacter species, notably *Campylobacter jejuni* and *Campylobacter coli*, are the most commonly reported bacterial causes of human gastroenteritis in the world (1). They are widely distributed in nature (2), and ingestion of contaminated food or water is a major route of human infection (3). Identification of sources and infectious trends is often vital for informing control measures, and this process requires subtyping of isolates. A variety of subtyping methods are available for *Campylobacter*, with the most widely used being multilocus sequence typing (MLST) (4) and pulsed-field gel electrophoresis (PFGE) (5). Neither of these techniques is particularly rapid, with analysis times of several days (6, 7). Data processing can also be complex (7, 8).

With the aim of making subtyping of all *C. jejuni* isolates achievable for laboratories globally, our group previously developed an 18-target PCR binary typing (P-BIT) system for *C. jejuni* based on pathogenicity- or survival-associated genes that required no specialized laboratory resources (9). P-BIT does, however, require 18 individual PCRs per isolate. Multiplex ligation-dependent probe amplification (MLPA) is a modification of PCR that allows up to 40 targets to be detected in a single reaction (10). It requires a standard thermal cycler, and PCR products can be visualized using a range of detection platforms. This paper reports on the conversion of the P-BIT system to the MLPA-binary typing (MBiT) format, which allows all 18 products to be examined in a single reaction. We also demonstrate its utility in an outbreak setting.

MATERIALS AND METHODS

MLPA probes were designed for the 18 discriminatory gene targets used in the *Campylobacter* P-BIT system (9) according to the instructions provided by the pioneers of MLPA, MRC-Holland (Amsterdam, The Netherlands). The probes were manufactured as described by Schouten and colleagues (11) at MRC-Holland. The target genes, specific probe sequences, primer sequences, and product sizes are listed in Table 1.

Each of the 222 *C. jejuni*, 22 *C. coli*, and 1 *Campylobacter lari* isolates listed in Data Set S1 (in the supplemental material) were grown on Columbia sheep blood agar plates at 42°C under microaerobic conditions for 48 to 72 h. Suspensions were prepared in phosphate-buffered saline (BR0014G; Oxoid, Basingstoke, England) to a density of 0.8 using a tur-

bidity meter (Dade International, West Sacramento, CA), and DNA was extracted using the method described previously (9). Each DNA extract was diluted to 20 ng/μl in Tris-EDTA (TE) buffer (Life Technologies) and analyzed using the standard MLPA protocol, SALSA MLPA reagent kits (MRC-Holland), and *Campylobacter* MBiT probe mixes. The hybridization time was shortened from 16 h to 1 to 2 h. The MLPA products were diluted 1:100 in water and separated using the ABI genetic analyzer 3130XL with POP-7 polymer and GeneScan 600 LIZ size standard (Life Technologies). The data were analyzed using GeneMapper (Life Technologies). The presence or absence of each product was scored as 0 or 1 to make a binary code and converted to a 6-digit type, as described for the earlier P-BIT assay (9).

The ability of a variety of detection methods to differentiate MBiT products was evaluated using three fully sequenced *C. jejuni* isolates (NCTC11168, RM1221, and RM1864 [81-176]), one fully sequenced *C. coli* isolate (RM2228), and the control plasmids supplied with the *Campylobacter* MBiT probe mixes. Each sample was tested using probe mixes containing (i) all 18 probe pairs, (ii) the odd-numbered probe pairs, and (iii) the even-numbered probe pairs (Table 1). The remainder of the MLPA assay was performed as described above. The products were detected (i) diluted 1 in 100 in water on the ABI genetic analyzer as above, (ii) undiluted on the MCE-202 MultiNA microchip electrophoresis system (Shimadzu Corporation, Kyoto, Japan) using standard operating procedures for on-chip mixing with the DNA 1000 reagent kit (Shimadzu), and (iii) undiluted in a 2% agarose gel in 1× Tris-borate-EDTA (TBE) at 110 V for 70 min and visualized using ethidium bromide.

TABLE 1 Target genes, specific probe sequences, and primer sequences used in *Campylobacter* MBiT

No.	Target gene	Left probe oligonucleotide	Right probe oligonucleotide	Product size (nt)[a]
1	*tet*O	CAGATACAATGAATTTGGAGCGTCAAAGG	GGAATCACTATCCAGACAGCAGTGACATC	124
2	*vir*B8	GTAAAGGTAGAACAAGCAGGAATGGATATGAG	AGCCGATGAGAATTTATTAAAATCAATTCTCGCAGGCT	142
3	*cgt*A	CTTTGGATAGCAGGCAATATACTTTCAAGAGAA	GCTTTTAAGGTAATAACTTCATTTTTTACTCGTATAAAAGCCC	160
4	Cj1136	GTTTTTTGCCTCTTTAGCATTTTCTCCAAAAA	ATTCATACAAGTCTTTAAAATACTCTGACACATTTGCC	178
5	*pan*B	GATCGTGACGCTATCTTAAATGCCTCAAG	ATTTATCAAAGAAAGCCACGCAAATGGGGTAAAAGTGGAAG	196
6	*maf*5	CAGCCAGTTCATAAGCCATATGAGATACACTCATG	CCTGCGTTAATATATCCATAATCGTCTAGCCC	214
7	Cj1135	CGCTTTCATTTTTATTTAAAATTACTCTTGAACCTTGTAAATTACTTTG	CGTTTTGCAAATTCTAAATGATTTTTTACAAAGTCTTTTTCTAAAATCATATC	232
8	Cj0265	GGATTTACATAACCATCAATTTTTAGCAAAACCCTGTTA	TTTTCGCTTTTTAATACTTCAAAAGGATTTGTAGGTAAAAGTC	250
9	CJE1733	CTTTATGTCTTATTTTGATGATGTATCCGCCTTTG	GCTAAGGTTGATTATGCAAAGCTTTCAAAGGT	268
10	Cj0122	CTAAGCTCTTTTAATAGATATTTTGGAAACAATCCTTTGCAA	ACACTTACCAAAATAAGAGATGAAAGTATTGAAAATGGAAATC	286
11	*gmh*A2	GTTAATATGCTAGTATCTGTAGTAAGTGCAATACTTGCGATA	CCGGGTCTATCAAAATAAAACCTACTTACAAATTCCC	311
12	*flg*E2	GTTCTTCTTTGTGGACAGCTACAAATATTACATTTACAC	CACAACCTCCTCAAGCAGCTACGAATGT	338
13	CJE1500	GTTTTGATGATGGTCTAAAAGATCATTACGATTTCG	TTTTCCCTAAACTCTTAGAGCATAAAATTTTTGGATTATTTTTATACC	365
14	Cj0423	CTGGCTGATAATTTCTTTGCTAAATTTGTTTTTTGG	TTGGACTATTATTGTTTGGCTTGTTTGTTTAATATGGTCT	391
15	*wla*N	CCACAATCATCTACTACAATGATTTCTATATCTTTAAAGTTTGGTTA	ATGCAACTTTCTAATGCTCTAGCAATATATTTTTCCACAT	418
16	*cfr*A	CCAAAAAAGTAAGTGATAAATGGGAAACTTCTGTAAG	TTTGGATGCTCTTTTAAATGAAAATAAAGATTGGGGTAATACTT	445
17	Cj1321	CATGTAATTTTATCACTTTTATACATCCTCAATCTTTTGTTTCA	AAAGAAGCTAAAATAGGACAAGGGGTTATAGTGTGTGT	473
18	Cj0008	GAAAATAATGTCATAGAATGCCGTAAAAATAGTCCTGAAG	CGTTTGCTGTGTTATCTTTACTTTATCCAAATTTAGATTATAAAAATAATAATTTT	503
	SALSA PCR primers	FAM-GGGTTCCCTAAGGGTTGGA	GTGCCAGCAAGATCCAATCTAGA	NA[b]

[a] nt, nucleotides.
[b] NA, not applicable. SALSA PCR primers are used to amplify all MLPA probes once the left and right probe oligonucleotides are ligated.

For comparison, *C. jejuni* and *C. coli* strains were also analyzed using *C. jejuni* multilocus sequence typing (MLST) (12). Amplification was performed in a 25-μl volume using AmpliTaq Gold master mixture (Life Technologies) and 5 pmol of each primer. Products were sequenced with the ABI genetic analyzer and alleles were assigned using the *Campylobacter* PubMLST database (http://pubmlst.org/campylobacter/) (13). Novel alleles and sequence types (ST) were submitted for clonal complex (CC) designations.

To demonstrate the usefulness of MBiT in an outbreak setting, 51 human *C. jejuni* and *C. coli* isolates recovered by Christchurch clinical laboratories over the period in which an outbreak of waterborne gastroenteritis was occurring in the nearby town of Darfield were subtyped using PFGE (14) and MBiT. Either a single colony from the primary isolation plate or a small section of a PFGE plug (1/8 of the size used for PFGE) was added to 5 μl of TE buffer and used as the sample for MBiT analysis.

RESULTS

Strain MBiT profiles derived from the genetic analyzer or MultiNA systems were consistent for all samples and probe mixes. MBiT products, with length differences as small as 18 nucleotides, were detectable in as little as 5 min with the MultiNA. Furthermore, the individual MLPA were distinguished using agarose gel electrophoresis when the odd- and even-numbered probes were tested in separate tubes (see Fig. 1 for examples).

From the 245 strains examined, MBiT produced 120 types with a Simpson's index of diversity (ID) (http://darwin.phyloviz.net/ComparingPartitions) of 0.987. Using MLST, 105 sequence types (ID, 0.979) and 26 clonal complexes (ID, 0.897) were identified.

To compare genomic differences between recently isolated human *C. jejuni* and *C. coli* that are geographically distant, we compared MBiT results for the New Zealand strains isolated in 2009 with the Belgian strains isolated in 2010. Of interest, *tet*O, which encodes tetracycline resistance, and CJE1733, which encodes an arsenical-resistance protein (provisional), were observed more frequently in Belgian isolates than New Zealand isolates (Belgium, 40 isolates; New Zealand, 4 isolates [Fisher Exact, 2-tailed $P = 0$] and Belgium, 42 isolates; New Zealand, 25 isolates [$P = 0.014$], respectively). Conversely, Cj1135, a putative two-domain glycosyltransferase, was observed more frequently in isolates from New Zealand (Belgium, 27 isolates; New Zealand, 47 isolates [$P = 0.041$]).

Of the 51 human *C. jejuni* and *C. coli* isolates received by the Institute of Environmental Science and Research (ESR) from Christchurch clinical laboratories during the Darfield waterborne gastroenteritis outbreak, 27 (representing 25 cases) were isolated from cases with home addresses in Darfield. The majority (22/27,
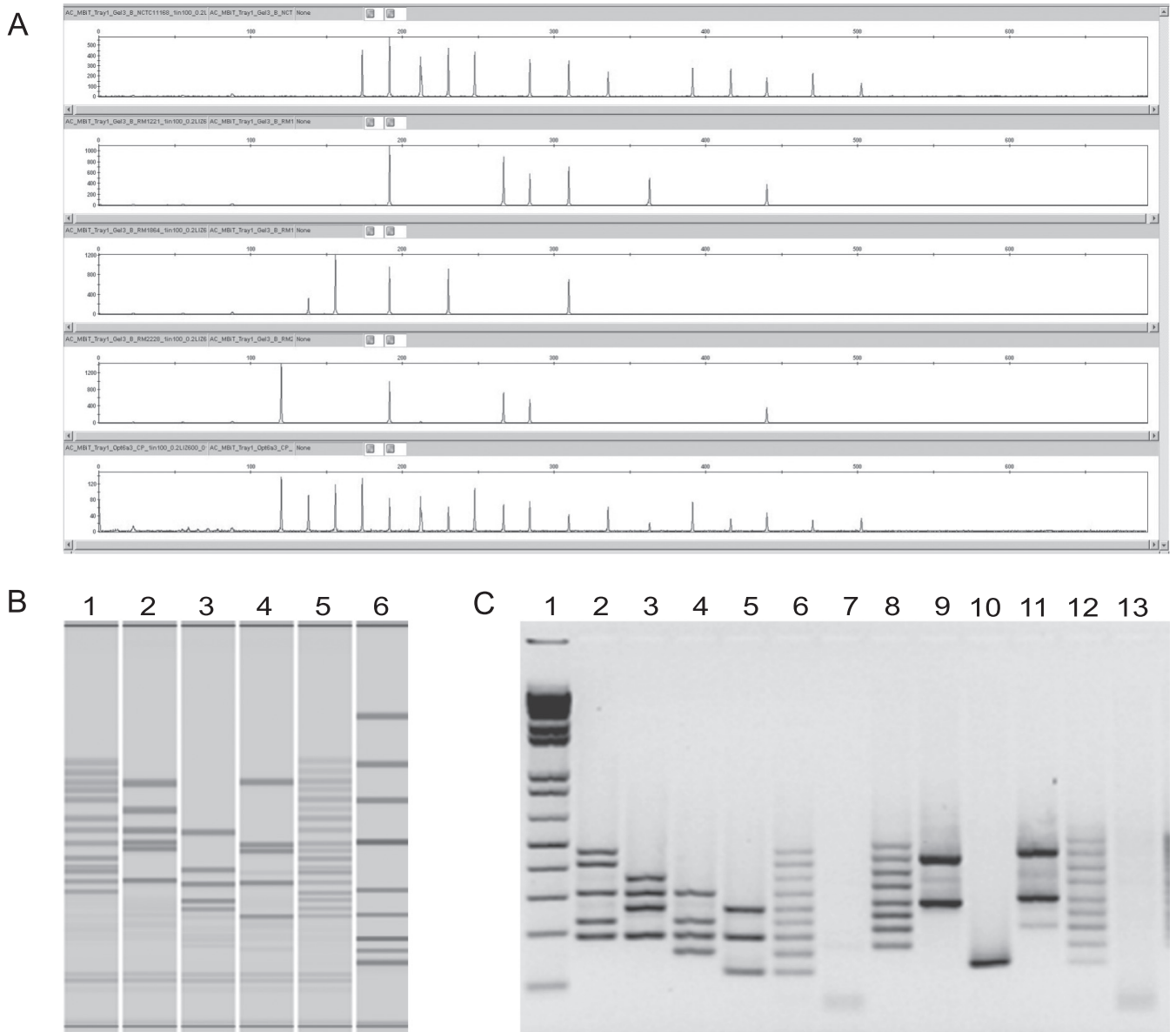
FIG 1 Illustration of alternative *Campylobacter* MBiT detection methods using fully sequenced isolates and control plasmids. In each image the order is NCTC11168 (MBiT 073767), RM1221 (MBiT 024311), RM1864 (MBiT 621200), RM2228 (MBiT 164101), and control plasmids (MBiT 777777). (A) ABI genetic analyzer; (B) MultiNA with lane 1, Fermentas GeneRuler Low Range DNA Ladder; (C) agarose gel electrophoresis with lane 1, Life Technologies 1 kb Plus DNA Ladder; lanes 7 and 13, TE; lanes 2 to 7, odd-numbered probes only; lanes 8 to 13, even-numbered probes only.

81%) of the Darfield isolates were *C. coli* with the Sm0131:Kp0132 PFGE profile and 064103 MBiT type, 1 was a *C. coli* with the Sm0431:Kp0564 PFGE profile and 024101 MBiT type, and the remaining 4 were *C. jejuni* with different PFGE profiles and MBiT types (Fig. 2).

Isolates from two cases that did not live in Darfield were *C. coli* with the outbreak PFGE profile and MBiT type. One of these cases, with a Christchurch address, was contacted by a local health protection officer, and it was confirmed that this individual had been working in Darfield and drinking from the town water supply during the exposure period of the outbreak. The other case with the outbreak PFGE profile and MBiT type was not contacted. Three clusters of 2 to 4 strains, each with indistinguishable PFGE and MBiT profiles, from Ashburton or Christchurch cases were

also identified. Two other clusters of 2 to 3 strains with indistinguishable profiles comprised cases from different geographical locations.

DISCUSSION

This pilot study demonstrates that MBiT achieved several of the performance criteria first proposed by Struelens (15) and updated by van Belkum and colleagues (16). It has high typeability, with all 245 isolates producing positive results for at least one target gene. It has high discriminatory power, with an ID greater than that of MLST. It has clear applicability to the investigation and identification of outbreaks. *Campylobacter* MBiT produces results within 6 h and uses readily accessible reagents, equipment, and skills. For laboratories with access to multichannel pipettes and automated
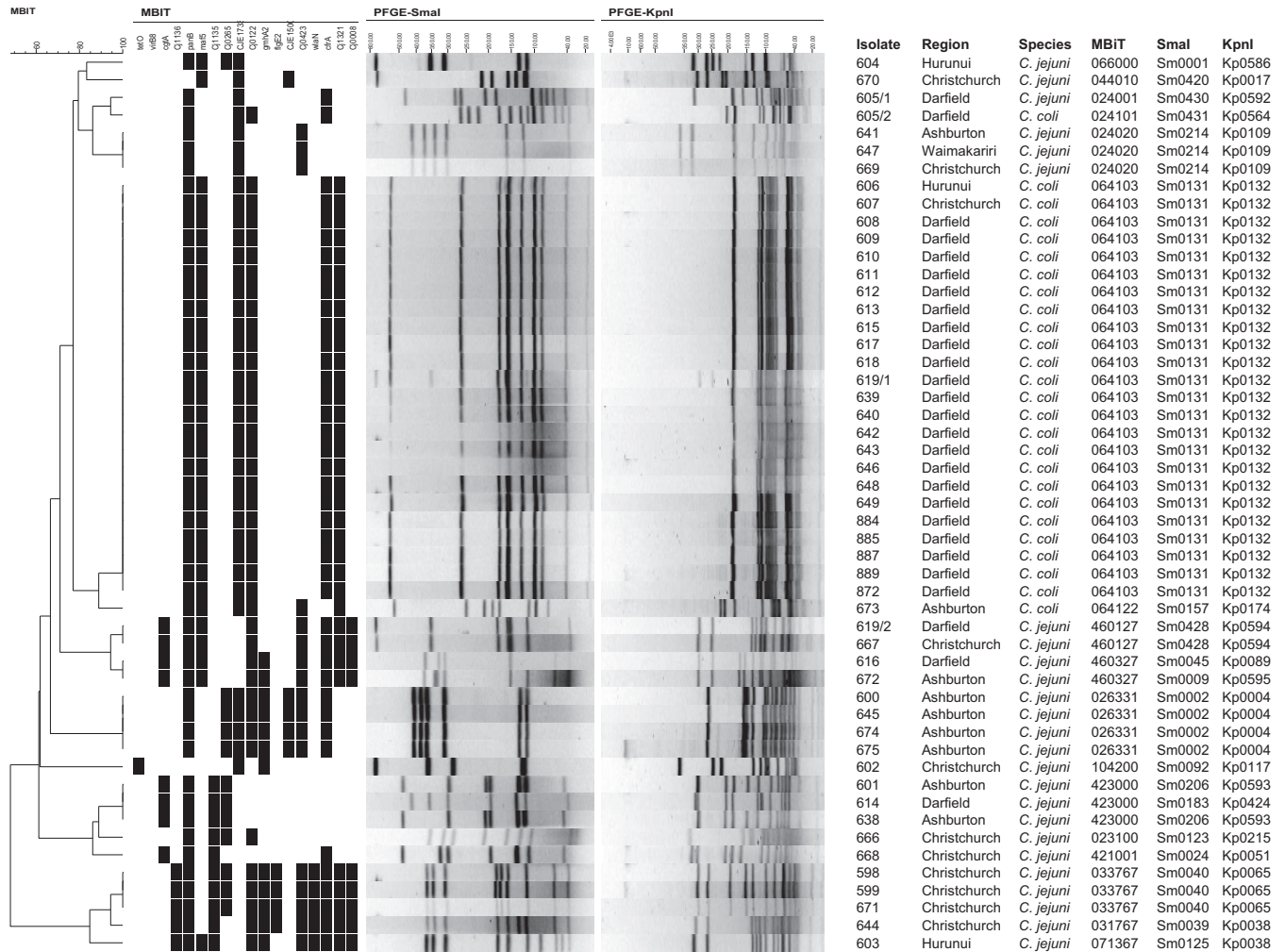
FIG 2 Cluster analysis of *Campylobacter* MBiT data for the isolates submitted during the Darfield waterborne gastroenteritis outbreak, prepared using simple matching and unweighted-pair group method with arithmetic mean (UPGMA).

electrophoresis equipment, such as the genetic analyzer and MultiNA, the *Campylobacter* MBiT assay offers an easy, single-tube assay with the potential for high-throughput subtyping of large numbers of isolates. The two-tube testing option required for detection by gel electrophoresis is still easy to perform but uses more reagents and consumables. It also requires controls to run more frequently in order to compensate for fragment migration fluctuations on the gel. Thus, the two-tube system is more expensive than the single-tube option and has limited throughput capability. Nonetheless, by designing the *Campylobacter* MBiT assay with single- and two-tube options, we have designed a system that is accessible to a wide range of laboratories from (i) reference laboratories with expensive equipment, software, and highly skilled practitioners testing hundreds of isolates a week to (ii) front-line public health laboratories, food industry laboratories, and laboratories in developing countries that are likely to have limited equipment, practitioners with less-developed skills, and fewer isolates to test. The six-digit MBiT code is highly portable and also amenable to computerized analysis and incorporation into electronic databases. All of these features make MBiT a very convenient subtyping system (16). In addition to these performance and convenience criteria, the choice of virulence- and sur-

vival-associated genes means the *Campylobacter* MBiT assay may generate data useful for molecular risk assessment (8) and evaluation of the genetic effects of intervention strategies or changes in industry practices over time.

The differences in carriage of the antibiotic resistance genes *tet*O and CJE1733 in human isolates from Belgium and New Zealand reflects the prevalence of antimicrobial resistance phenotypes in the two countries. Of the 318 human *Campylobacter* isolates tested in New Zealand during 2009, none were resistant to erythromycin and 5 (1.6%) were resistant to fluoroquinolone (https://surv.esr .cri.nz/PDF_surveillance/Antimicrobial/AR/National_AR_2009 .pdf). Conversely, of a set of 354 human *Campylobacter* isolates tested in Belgium during 2010, 11 (3.1%), 186 (52.5%), and 204 (57.6%) were resistant to erythromycin, ciprofloxacin (a fluoroquinolone), and ampicillin, respectively (17). Similar differences in antimicrobial resistance rates have been observed in isolates from meat and poultry in the two countries (17, 18).

The carriage of Cj1135, which encodes an enzyme that may be involved in the production of surface-associated sugars, may reflect bacterial cell surface features that increase its ability to infect human intestinal cells and thus may be partially responsible for the high rate of campylobacteriosis in New Zealand (1). Interestingly, Cj1135 has

been found to have a higher carriage rate among enteritis-associated strains from The Netherlands, Curaçao, and Japan (19), suggesting a possible human intestinal cell-specific interaction.

The utility of the MBiT assay in outbreak settings was observed in real time during the waterborne gastroenteritis outbreak in Darfield. MBiT results for the first set of samples received by ESR were available early the following morning. These results demonstrated that the majority of isolates from Darfield residents had a single MBiT type and that a Christchurch resident also had the outbreak MBiT type. Rapid follow-up by a health protection officer was possible and confirmed the patient had consumed Darfield town water during the exposure period, and so this case was formally included in the outbreak. PFGE results, which were available 2 days later, confirmed the MBiT results.

Although the P-BIT assay, from which the *Campylobacter* MBiT was adapted, was designed for *C. jejuni*, it appears also to provide useful subtyping information for *C. coli*, as demonstrated by the 12 different MBiT types observed for the 22 *C. coli* isolates in the validation study and the ability to identify the predominant type in an outbreak involving *C. coli*. Different MBiT types have so far been observed for *C. jejuni* and *C. coli* isolates. The *C. lari* isolate from the P-BIT paper was also typeable using the *Campylobacter* MBiT assay. The utility of the assay on *Campylobacter* sp. other than *C. jejuni* and *C. coli* is yet to be established. In future developments of the *Campylobacter* MBiT assay, we aim to identify targets that provide additional subtyping information for *C. coli* and potentially other *Campylobacter* sp.

The identification of several other small clusters of cases with indistinguishable PFGE profiles and MBiT types suggests that small outbreaks may be relatively common. No follow-up investigation was undertaken to identify common infection sources for these small clusters of cases. A small 2002 study of human cases in the same catchment area as the current study also observed numerous small clusters of cases with indistinguishable PFGE profiles (20). A larger 2009 to 2010 study in the same geographical location (21), which also observed clustering of cases based on PFGE, demonstrated how, even with typing data from human, environmental, and food-related isolates, source identification can be difficult. The authors of that paper suggested that a more rapid subtyping method, with turnaround time measured in hours rather than days, would allow more efficient use of epidemiological resources (21), thus facilitating a more rapid response with consequent benefits to public health and economics. Monitoring of small clusters may also facilitate the identification of point sources linked to recurrent events that, taken together, represent significant numbers of human cases and thus are worthy of additional public health resources and interventions. An example would be a producer, supplier, or restaurant linked to several small clusters of divergent types over a period of several months. MBiT is rapid and cheap enough that, for the first time, subtyping of all *C. jejuni*/*C. coli* isolated from human cases is possible. This will make it possible to use subtyping data alongside, or potentially to focus, epidemiological data collection to identify, investigate, and monitor clusters of all sizes.

In conclusion, the speed and cost of the *Campylobacter* MBiT assay make it an ideal candidate for use as a front-line subtyping system for both industrialized and developing countries and make possible the real-time subtyping of all *C. jejuni* and *C. coli* isolates. This assay is commercially available from MRC-Holland.

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