JOURNAL OF CLINICAL MICROBIOLOGY, July 2009, p. 1996–2007 Vol. 47, No. 7
0095-1137/09/$08.00+0 doi:10.1128/JCM.00237-09


# Multiplex Strategy for Multilocus Sequence Typing, *fla* Typing, and Genetic Determination of Antimicrobial Resistance of *Campylobacter jejuni* and *Campylobacter coli* Isolates Collected in Switzerland▽

Bożena M. Korczak,* Monika Zurfluh, Stefan Emler, Jacqueline Kuhn-Oertli, and Peter Kuhnert

*Institute of Veterinary Bacteriology, University of Bern, Bern, Switzerland, and SmartGene, Zug, Switzerland*

Received 4 February 2009/Returned for modification 3 April 2009/Accepted 5 May 2009

**We present an optimized multilocus sequence typing (MLST) scheme with universal primer sets for amplifying and sequencing the seven target genes of *Campylobacter jejuni* and *Campylobacter coli*. Typing was expanded by sequence determination of the genes *flaA* and *flaB* using optimized primer sets. This approach is compatible with the MLST and *flaA* schemes used in the PubMLST database and results in an additional typing method using the *flaB* gene sequence. An identification module based on the 16S rRNA and *rpoB* genes was included, as well as the genetic determination of macrolide and quinolone resistances based on mutations in the 23S rRNA and *gyrA* genes. Experimental procedures were simplified by multiplex PCR of the 13 target genes. This comprehensive approach was evaluated with *C. jejuni* and *C. coli* isolates collected in Switzerland. MLST of 329 strains resulted in 72 sequence types (STs) among the 186 *C. jejuni* strains and 39 STs for the 143 *C. coli* isolates. Fourteen (19%) of the *C. jejuni* and 20 (51%) of the *C. coli* STs had not been found previously. In total, 35% of the *C. coli* strains collected in Switzerland contained mutations conferring antibiotic resistance only to quinolone, 15% contained mutations conferring resistance only to macrolides, and 6% contained mutations conferring resistance to both classes of antibiotics. In *C. jejuni*, these values were 31% and 0% for quinolone and macrolide resistance, respectively. The *rpoB* sequence allowed phylogenetic differentiation between *C. coli* and *C. jejuni*, which was not possible by 16S rRNA gene analysis. An online Integrated Database Network System (SmartGene, Zug, Switzerland)-based platform for MLST data analysis specific to *Campylobacter* was implemented. This Web-based platform allowed automated allele and ST designation, as well as epidemiological analysis of data, thus streamlining and facilitating the analysis workflow. Data networking facilitates the exchange of information between collaborating centers. The described approach simplifies and improves the genotyping of *Campylobacter*, allowing cost- and time-efficient routine monitoring.**

Infection with *Campylobacter* has become the major cause of bacterial enteritis in Europe and other parts of the developed world, overtaking *Salmonella* infection (8). *Campylobacter jejuni* accounts for approximately 90% of all *Campylobacter* infection cases, whereas *C. coli* is responsible for approximately 10% of infections. Other *Campylobacter* species, such as *C. lari*, *C. upsaliensis*, *C. hyointestinalis*, and *C. fetus*, are sporadically found (24). Due to the fact that *Campylobacter* is mostly commensal in the enteron of many warm-blooded animals used for meat production, campylobacteriosis is a zoonotic disease. Quality control, monitoring, and eventually tracing of contaminated food products is therefore important for public health reasons. *Campylobacter* typing by applying various, mostly genetic, methods is used for this purpose. Classical pulsed-field gel electrophoresis and amplified fragment length polymorphism, as well as *flaA* typing based on the restriction analysis of PCR-amplified fragments or sequencing of the flagellin-encoding gene, have been described for *Campylobacter* (20, 37). Recently, multilocus sequence typing (MLST) has been established as a highly reproducible method allowing precise and simple worldwide comparison of types, and it is becoming the gold standard in this field (4–6,13,17–19, 22, 23, 30, 33). Despite its many advantages, MLST is still time-consuming and expensive and therefore not feasible for routine testing. For example, the scheme for *C. jejuni* typing recommended by the PubMLST database hosted by the University of Oxford, Oxford, United Kingdom (http://pubmlst.org/campylobacter) includes a total of 51 different primers to be used for PCR amplification and sequencing of the seven target gene sequences. Another 14 primers are described for MLST of *C. coli*. With problematic isolates, optimal primer combinations have to be determined, and reactions have to be repeated in order to obtain all seven allele sequences needed for sequence type (ST) determination.

MLST alone provides excellent information about the global epidemiology and population structure of *Campylobacter*, but it appears to be less discriminative in short-term epidemiological studies (28). The addition of more variable targets, such as flagellin-encoding genes, increases the discriminatory power of sequence-based typing. The most frequently used gene for this purpose is *flaA* (2, 5, 7, 17, 20, 26, 29), although *flaB* is also used, and as a more stable gene, *flaB* might become more important (21). Other important factors to consider are the time and effort needed to perform the appropriate data analysis, especially in the context of internationally standardized approaches and the use of publicly available typing tools, such as http://pubmlst.org.

Since the 1990s, the prevalence of antibiotic resistance has

* Corresponding author. Mailing address: Institute of Veterinary Bacteriology, University of Bern, Laenggass-Str. 122, CH-3001 Bern, Switzerland. Phone: 0041 (0)31 631 24 91. Fax: 0041 (0)31 631 26 34. E-mail: bozena.korczak@vbi.unibe.ch.

▽ Published ahead of print on 13 May 2009.

increased dramatically in both animal and human *Campylobacter* isolates. This is especially the case for quinolone resistance, the emergence of which is correlated with the introduction of quinolones in the treatment of food-producing animals. The emergence of macrolide-resistant *Campylobacter* isolates has also been observed but until recently was less pronounced than quinolone resistance (41). Quinolone resistance is mainly based on a point mutation in the gyrase gene, *gyrA* (C257T or, less frequently, A256G) (1). In the case of macrolide resistance, it is caused by a point mutation (A2075G or A2074C) in the loop in domain V of the 23S rRNA gene (34).

In order to optimize and simplify the amplification and sequencing strategy for MLST and combine it with sequence-based *fla* typing, as well as with antibiotic resistance determination, we established a modular and adaptable multiplex PCR and sequencing protocol using the minimum number of primers, which can be used equally well for *C. jejuni* and *C. coli*. About 95% of human *Campylobacter* infections can be covered with our typing scheme. Proper identification of *Campylobacter* isolates is not always trivial, and misidentification might hamper downstream typing, especially genotyping. 16S rRNA and *rpoB* genes were included in the multiplex approach as a basic genetic identification module for the genus *Campylobacter*, and the discriminatory power at the species level was examined. Through this approach, enteritis-causing *Campylobacter* species other than *C. jejuni* and *C. coli* are dealt with by proper identification.

The robustness of the multiplex approach was tested on more than 300 *C. jejuni* and *C. coli* strains. Data analysis was performed using a newly developed Internet-based Integrated Database Network System (IDNS) (SmartGene, Zug, Switzerland) platform for genotyping *Campylobacter*.

## MATERIALS AND METHODS

**Isolates and growth conditions.** Phenotypically characterized *C. jejuni* (180) and *C. coli* (141) isolates from the collection at the Institute of Veterinary Bacteriology, University of Bern, Bern, Switzerland, were used. The strain set contained human isolates from patients suffering from clinical campylobacteriosis sampled between 1993 and 2003 at the Swiss National Reference Centre for Enteropathogenic Bacteria, cattle isolates collected from healthy dairy cows during December 2001 and January 2002, pet isolates collected from healthy dogs and cats from 2002 to 2003, and poultry and pig isolates collected in 2002 (27, 38, 40). The isolates originated from different geographical regions across Switzerland. The type strains of *C. jejuni* NCTC 11351$^T$, *C. jejuni* subsp. *doylei* LMG 8843$^T$, and *C. coli* LMG 6440$^T$, as well as the *C. jejuni* reference strains ATCC 29428, CCUG 10937, CCUG 12066, and NCTC 11168 and *C. coli* CCUG 12068, were included in the analysis.

The isolates were stored at −80°C until they were cultivated on tryptone soya agar plates with sheep blood (Oxoid, Hampshire, United Kingdom) for 24 to 48 h at 42°C under microaerophilic conditions.

To examine the genetic stability of the strains, NCTC 11168 and one poultry field isolate were serially passaged in vitro in nonselective medium. For each strain, one colony from an agar plate was inoculated into a 100-ml Erlenmeyer flask containing 25 ml Mueller-Hinton broth (Difco, Becton Dickinson, Sparks, MD) supplemented with 5% lysed horse blood (Oxoid) and incubated at 37°C under microaerophilic conditions. After 24 h, 250 μl of culture, which was in exponential phase, was transferred into 25 ml of fresh medium and incubated under the same growth conditions. The number of cells in each 24-h-old culture was determined by measurement of the optical density at 600 nm. This step was repeated 30 times for NCTC 11168 and 38 times for the poultry isolate, which is equivalent to about 200 and 250 generations, respectively. Material from the first and the last liquid culture was used to sequence the target genes.

**Isolation of genomic DNA.** Extraction of total DNA was performed using either the E.Z.N.A. Bacterial DNA kit (Peqlab Biotechnologie GMBH, Erlangen, Germany) according to the manufacturer's instructions or by simple lysis of the bacteria. For this purpose, a few bacterial colonies from the plates were resuspended in 450 μl of lysis buffer (0.1 M Tris-HCl, pH 8.5, 0.05% Tween 20, 240 μg/ml proteinase K), incubated at 60°C for 1 h, and heat inactivated at 94°C for 15 min. The extracted DNA and lysates were stored at −20°C until they were used.

**Selection of conserved and optimized primers.** Based on the currently available genome sequences of *C. jejuni* and *C. coli*, conserved primer sequences were defined which match the criteria for PCR amplification and sequencing. These primers were chosen to cover the classical gene regions used for allele determination in the Oxford scheme (PubMLST). Primer sets were designed for both the *flaA* and *flaB* genes, covering the "short variable regions," and for the amplification of the *gyrA* fragment. Previously published primers were used to generate the fragments from the 23S rRNA gene (34), the 16S rRNA gene (16), and the *rpoB* gene (15). The primers and their locations, as well as the resulting fragment sizes, are listed in Table 1.

**Multiplex PCR amplification and purification.** Multiplex PCR was established in order to facilitate and economize laboratory work. For this purpose, primers for the amplification and sequencing of 13 targets per strain (Table 1) were divided into four amplification groups (AGs), taking into account the PCR product length and amplification efficiency for all targets. The first group (AG1) contained primers for *flaA* (flagellin), the 23S rRNA gene, *aspA* (aspartase), and *glmM* (also called *pgm* for phosphoglucosamine mutase); the second group (AG2) contained primers for *gyrA* (gyrase), *flaB* (flagellin), *tkt* (transketolase), and *glnA* (glutamine synthetase); and the third group (AG3) contained primers for *gltA* (citrate synthase), *atpA* (also called *uncA*; the ATP synthase α subunit), *glyA* (serine hydroxymethyltransferase), and *rpoB* (the β subunit of the RNA polymerase). A fourth group (AG4) was comprised of universal primers for the amplification of the 16S rRNA gene (16S UNI-L and 16S UNI-R). Each reaction was performed in a 30-μl total volume containing 12 pmol of each primer, 0.25 mM dATP, 0.25 mM dCTP, 0.25 mM dGTP, 0.25 mM dTTP (Roche, Rotkreuz, Switzerland), 5 mM $MgCl_2$ (2.5 mM in the case of 16S rRNA), 1× reaction buffer, 2.5 U Fire Pol DNA polymerase I (Solis BioDyne, Tartu, Estonia), and approximately 50 ng DNA. Each PCR was run in a 9800 Fast Thermal Cycler (Applied Biosystems, Foster City, CA) under the following universal conditions: 3 min of denaturation at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 56°C, and 1 min at 72°C and a final extension step at 72°C for 7 min. Multiplex PCR products (3.0 μl from each AG) were analyzed on a 1.5% agarose gel stained with 0.3 μg/ml ethidium bromide. Bands of similar intensity indicated equally efficient amplification of the specific products. To enzymatically purify the samples from residual deoxynucleotides and excess primers, 8.0 μl of the AG1, AG2, or AG3 PCR product and 4.0 μl of the AG4 PCR product was transferred into new reaction tubes, followed by the addition of 1.0 μl rAPid Alkaline Phosphatase (1 U/μl; Roche Diagnostics), 0.2 μl of the corresponding buffer, and 0.05 μl exonuclease I (*Exo* I; 20 U/μl; New England Biolabs, Ipswich, MA). The samples were incubated in the 9800 Fast Thermal Cycler (Applied Biosystems) for 30 min at 37°C and then for 20 min at 80°C to inactivate the enzymes.

**Sequencing of multiplex PCR products.** The purified PCR products were directly sequenced using the same primers used for PCR. For the 16S rRNA gene fragment, additional internal primers (16S RNA2-S, 16S RNAII-S, and 16S RNA6-S) were used. A total of 28 sequencing reactions for each strain were necessary. For convenient handling during the preparation of these numerous reactions, 96-well plates and reaction tube strips were prepared in advance by adding primers. To prepare the plates and strips, 1.0 μl containing 5 pmol of the specific primer was pipetted onto the bottom of the corresponding well or tube using a multichannel pipette and then dried at room temperature. The sequencing plates and strips were then stored at −20°C until they were used. For each strain, four sequencing mixtures were directly prepared in the tubes containing the purified multiplex PCR product by adding 8.0 μl (AG1, AG2, and AG3) Big Dye v3.1, 8.0 μl (AG1, AG2, and AG3) sequencing buffer (Applied Biosystems), and 16.0 μl (AG1, AG2, and AG3) double-distilled $H_2O$. For the purified 16S rRNA PCR product, 4.0 μl Big Dye v3.1, 4.0 μl sequencing buffer, and 8.0 μl double-distilled $H_2O$ were added. Next, 5 μl of this mixture was added to the corresponding wells or tubes on the sequencing plate or strip, respectively. Cycle sequencing was performed in a GeneAmp PCR System 9700 (Applied Biosystems) with 25 cycles of 10 s at 96°C, 5 s at 50°C, and 1 min at 60°C. The products were purified by ethanol precipitation by adding 100 μl 0.5 mM $MgCl_2$/60% ethanol and centrifuging them for 40 min at 4,500 rpm (3,840 × *g*) at 10°C using a Rotanta 46R centrifuge (Hettich Zentrifugen GmbH & Co., Tuttlingen, Germany). The supernatant was discarded, and the sequencing plates/strips were inverted and centrifuged at 1,000 rpm (190 × *g*) for 1 min to dry the samples. The samples were run on an ABI Prism 3130*xl* Genetic Analyzer (Applied Biosystems).

TABLE 1. Primers used for PCR and sequencing of MLST and *fla* genes of *C. jejuni* and *C. coli*

| AG | Gene product | Primer name[a] | Sequence (5′→3′) | Positions on NCTC 11168 (accession no. AL111168) | PCR fragment size (bp) | Source |
|---|---|---|---|---|---|---|
| 1 | Flagellin | flaA_Cjc-L | TAATACTTTAGGTCAAGCTATATC | 1270777–1270754 | 471 | This study |
| | | flaAB_Cjc-R | CCAAGWCCTGTTCCWACTGAAG | 1270307–1270328 | | Taken from reference 21 |
| | Alternative forward primer | flaA_Cjc-L1 | GCTTTAAATGCAAAAGCAAACGC | 1268443–1268464 | 614 | This study |
| | 23S rRNA gene | F1-campy-23S | AAGAGGATGTATAGGGTGTGACG | 43392–43414 | 508 | Taken from reference 34 |
| | | R1-campy-23S | AACGATTTCCAACCGTTCTG | 43899–43880 | | |
| | Aspartase | aspA_Cjc-L | CAACTKCAAGATGCWGTACC | 96635–96654 | 594 | Adapted from reference 5 |
| | | aspA_Cjc-R | ATCWGCTAAAGTATRCATTGC | 97228–97208 | | Adapted from reference 5 |
| | Phosphoglucosamine mutase | glmM_Cjc-L | GCTTATAAGGTAGCWCCKACTG | 327701–327722 | 685 | Adapted from reference 30 |
| | | glmM_Cjc-R | AATTTTCHGTTCCAGAATAGCG | 328385–328364 | | Adapted from reference 23 |
| 2 | Gyrase | gyrA_Cjc-L | GAGYGTTATTATMGGTCGTGC | 960136–960116 | 294 | Adapted from reference 14 |
| | | gyrA_Cjc-R1 | TCAGTATAACGCATCGCAGC | 959843–959862 | | This study |
| | Flagellin | flaB_Cjc-L | TCTTTGCGTTCACAAGCAGC | 1268930–1268911 | 488 | This study |
| | | flaAB_Cjc-R | CCAAGWCCTGTTCCWACTGAAG | 1270307–1270328 | | Taken from reference 21 |
| | Alternative forward primer | flaB_Cjc-L1 | CATGCAAATTCAGTTGTTAATGC | 1268443–1268464 | 602 | This study |
| | Transketolase | tkt_Cjc-L | AAAYCCMACTTGGCTAAACCG | 1569342–1569362 | 606 | This study |
| | | tkt_Cjc-R | TGACTKCCTTCAAGCTCTCC | 1569947–1569928 | | Adapted from reference 5 |
| | Glutamine synthetase | glnA_Cjc-L | ACWGATATGATAGGAACTTGGC | 658250–658229 | 712 | This study |
| | | glnA_Cjc-R | GYTTTGGCATAAAAGTKGCAG | 657541–657559 | | Adapted from reference 5 |
| 3 | RNA polymerase β subunit | CamrpoB-L | CCAATTTATGGATCAAAC | 440572–440589 | 524 | Taken from reference 15 |
| | | RpoB-R | GTTGCATGTTNGNACCCAT | 441095–441077 | | |
| | Citrate synthase | gltA_Cjc-L | TATCCTATAGARTGGCTTGC | 1605035–1605016 | 567 | This study |
| | | gltA_Cjc-R | AAGCGCWCCAATACCTGCTG | 1604469–1604488 | | Adapted from reference 5 |
| | ATP synthase α subunit | atpA_Cjc-L | CAAAAGCAAAGYACAGTGGC | 112085–112104 | 623 | This study |
| | | atpA_Cjc-R | CTACTTGCCTCATCYAAATCAC | 112707–112686 | | Adapted from reference 5 |
| | Serine hydroxyl-methyltransferase | glyA_Cjc-L | AGGTTCTCAAGCTAATCAAGG | 367494–367514 | 701 | Adapted from reference 23 |
| | | glyA_Cjc-R | CATCTTTTCCRCTAAAYTCACG | 368194–368173 | | This study |
| 4 | 16S rRNA | 16S UNI-L | AGAGTTTGATCATGGCTCAG | 39258–39277 | 1,381 | Taken from reference 16 |
| | | 16S UNI-R | GTGTGACGGGCGGTGTGTAC | 40638–40619 | | |
| | Not applicable[b] | 16S RNA2-S | GTGTAGGGGTAAAATCCGTAG | 39907–39927 | | |
| | | 16S RNAII-S | GTGTAGCGGTGAAATGCGTAG | 39907–39927 | | |
| | | 16S RNA6-S | CTACGGATTTTACCCCTACAC | 39927–39907 | | |

[a] L, forward primer; R, reverse primer.
[b] Internal sequencing primers.

**Sequence analysis.** Sequence data were entered, edited, and analyzed in a newly established Web-based MLST application for *Campylobacter* identification, typing, and antibiotic resistance determination. This application was developed on the basis of a proprietary application service technology, IDNS, which combines target-specific semiautomated sequence editing, bioinformatics, and databases for *Campylobacter* MLST. Trace files were imported, automatically trimmed, and aligned to a best-match reference sequence in the Proofreader module of the software. The edited gene sequences were submitted via an integrated link to the public PubMLST typing site (http://pubmlst.org/campylobacter). Allele numbers, ST determinations, and clonal complexes (CC) were electronically recovered and made available as searchable results in the database.

Alternatively, sequences were edited in Sequencher (GeneCodes, Ann Arbor, MI) and entered into BioNumerics software version 5.1 (Applied Maths NV, Sint-Martens-Latem, Belgium). Cluster analysis of full-length sequences and calculations of the congruency of the experiments were performed using BioNumerics. The discriminatory abilities of *flaA* and *flaB* were calculated using Simpson's index of diversity (12).

**Antibiotic MIC determination.** Point mutation-based quinolone and macrolide resistances were compared with the actual phenotype. For most *C. coli* strains, MICs were available from a previous study (14). For the remaining *C. coli* and *C. jejuni* strains believed to be resistant based on their genetics, the MICs were determined using the Sensititre system (Trek Diagnostic Systems, England) according to the Clinical and Laboratory Standards Institute guidelines (3).

For this experiment, ciprofloxacin and erythromycin, representing the quinolone and macrolide classes of antibiotics, respectively, were used. The selected strains were tested in antibiotic concentrations of 0 (control culture), 0.5, 1, 2, 4, and 8 μg/ml. The cutoffs for ciprofloxacin and erythromycin resistance were set at ≥4 μg/ml and ≥8 μg/ml, respectively.

## RESULTS

**Multiplex PCR and sequencing.** The analysis of 329 *C. jejuni* and *C. coli* strains, including the type and reference strains of both species, was carried out by the new multiplex PCR and sequencing approach.

Simultaneous amplification of targets as AGs (AG1 to AG4) was achieved for all isolates with all seven MLST genes, as well as the *gyrA*, 23S rRNA, *rpoB*, and 16S rRNA genes. The sequences could be unambiguously determined from these multiplex reactions with the same primers and without interference from the amplification products.

The *flaB* fragment was amplified in all isolates, with the exception of the *C. jejuni* subsp. *doylei* LMG 8843$^T$ type strain. Analysis of the available genome sequence of *C. jejuni* subsp. *doylei* strain 269.97 (GenBank accession no. NC_009707) showed an absence of this gene, which might be characteristic of the subspecies.

Whereas the *flaA* fragment could be amplified in multiplex reactions from all *C. jejuni* strains, no amplification of *flaA* was obtained for one-third of the *C. coli* samples. Moreover, in a few strains of *C. coli* (6 and 20 strains, respectively) *flaA* and *flaB* sequencing resulted in ambiguous sequences, even though the amplification fragments were clear and the genes were

efficiently amplified. To solve these problems, the optional forward primers flaA_Cjc-L1 and flaB_Cjc-L1, for *flaA* and *flaB*, respectively, were designed (Table 1). The targets were amplified in separate reactions under the same conditions as the multiplex PCR and finally sequenced successfully.

After sequence editing and automatic truncation of the primers using the SmartGene software, the expected sizes for *aspA* (553 bp), *atpA* (581 bp), *glmM* (641 bp), *glnA* (669 bp), *gltA* (527 bp), *glyA* (658 bp), *tkt* (565 bp), *flaA* (425 bp), *flaB* (446 bp), *gyrA* (253 bp), 23S rRNA (465 bp), 16S rRNA (1,341 bp), and *rpoB* (487 bp) were obtained for each strain.

Potential artifacts resulting from multiple passaging of strains in the laboratory were evaluated in a small study analyzing the genetic stability of the typing genes (MLST, *flaA*, and *flaB*) over 200 to 250 generations. Both strains included in this validation, NCTC 11168 and the poultry field isolate, did not show any mutations during this extensive passaging.

**MLST and *fla* typing.** A total of 118 different STs, including the STs of the type and reference strains of *C. jejuni* and *C. coli*, were recognized in this study, 34 of which were new and had not been previously described. The MLST data for the isolates collected in Switzerland are summarized in Table 2. Within this set of isolates, the new STs were comprised of 14 STs specific for *C. jejuni* strains and 20 STs specific for *C. coli* strains. A total of 18 (10.0%) *C. jejuni* and 40 (28.4%) *C. coli* strains collected in Switzerland resulted in new STs.

In total, 61 STs of *C. jejuni* were distributed in 20 CCs. CC21 and CC45, comprising their respective STs, were predominant, containing 41 (22.8%) and 27 (15.0%) isolates, respectively, followed by CC48, which contained 16 (8.8%) isolates, and CC206, which contained 14 (7.7%) isolates. Eleven STs could not be assigned to any known lineage.

Nearly 90% of the *C. coli* isolates were distributed among 30 STs belonging to one CC, CC828, with ST854 predominant. The remaining strains represented nine STs that could not be assigned to any of the known CCs. The *C. coli* strains were more conserved in their types than the *C. jejuni* strains, with averages of 3.6 and 2.5 strains per ST, respectively.

The STs of the type strains of *C. jejuni* subsp. *jejuni* NCTC $11351^T$ (ST403), *C. jejuni* subsp. *doylei* LMG $8843^T$ (ST62), and *C. coli* LMG $6440^T$ (ST900) and the reference strains *C. jejuni* ATCC 29428 (ST50), CCUG 10937 (ST5), CCUG 12066 (ST267), and NCTC 11168 (ST43) have been confirmed, and in the case of *C. coli* strain CCUG 12068, a new ST2913 was assigned in the study.

For a few isolates belonging to the same ST, nucleotide differences were observed for some of the genes in the sequences flanking the regions used for allele designation by PubMLST. Within the *C. jejuni* isolates, the following differences could be seen: in ST45, transition A617G in the *glyA* fragment; in ST122, transition G10A in the *glyA* fragment; and in ST353, transitions C53T, T77C, and C127T and transversion T128A in the *glnA* fragment, as well as transition A76G in the *gltA* fragment. *C. coli* strains of ST2733 showed transitions C12T in the *glmM* fragment and T8C in the *tkt* fragment, while strains of ST3336 showed transition C12T in *glmM*. This is reflected in a slightly different branching in a composite tree built from the full-length sequences of *aspA*, *atpA*, *glmM*, *glnA*, *gltA*, *glyA*, and *tkt.* These results also allow further discrimination within certain STs compared to the classical PubMLST scheme (data not shown).

Typing based on partial *flaA* and *flaB* gene sequences was investigated by cluster analysis (Fig. 1 and 2). The similar clustering observed in *flaA* (124 branches) and *flaB* (107 branches) was reflected in a high congruence value of 98.5%. However, *flaA* provided greater discriminatory potential than *flaB*, as indicated by the Simpson's indexes of diversity of 0.855 and 0.799, respectively. Neither *flaA* nor *flaB* showed congruence with MLST (<5%). Nonetheless, in a sequence-based cluster analysis (data not shown), the addition of either *flaA* or *flaB* to MLST allowed closely related strains with the same ST to be further distinguished, and both genes increased the resolution of MLST. This is also reflected by the increase in the Simpson's index from 0.788 for MLST only to 0.958 for MLST combined with *flaA* and 0.968 for MLST combined with *flaB*.

**Antibiotic resistance.** Internal portions of the antibiotic resistance-related genes 23S rRNA and *gyrA* were amplified as part of AG1 and AG2, respectively, in all investigated strains. The point mutations in the 23S rRNA gene known to contribute to macrolide resistance, A2074G and A2075G (corresponding to A227G and A228G in our sequence fragment), were not observed in any of the *C. jejuni* strains (34). However, 20.6% of the *C. coli* isolates showed resistance to this group of antibiotics based on their 23S rRNA gene sequences. The transition A2075G was observed in 29 strains of *C. coli*, while A2074C was not found in any strain. Nearly all strains carrying the resistance-related mutation originated from pigs, with the exception of two resistant strains isolated from human feces and one strain from poultry. Interestingly, one human and three pig isolates showed an A-G double peak at nucleotide position 2075. The genetically derived resistance was confirmed by MIC resistance tests in the Sensititre system (MIC required for resistance, $\geq$8 μg/ml) for all cases, including the three strains with ambiguities at the crucial mutation position. Mutations at other positions in the 23S rRNA gene were present in a number of strains, but none of them conferred resistance as determined by the MIC tests.

Quinolone resistance, which is most often associated with the point mutation C257T (corresponding to C150T in our fragment) in the *gyrA* gene, was observed in 54 of the *C. jejuni* strains, while the transition A256G, less frequently reported to be a determinant of quinolone resistance, was found in two human isolates, resulting in 31% resistant strains. A large portion of these resistant strains were represented by human isolates (75%), followed by pet (12.5%), poultry (9%), and cattle (3.5%) isolates. Fifty-seven (40.4%) *C. coli* strains harbored the point mutation C257T, with all of them isolated from either pigs (87.7%) or humans (12.3%). Six percent of *C. coli* strains showed resistance to both classes of antibiotics.

Phylogenetic analysis of *gyrA* sequences revealed them to be species specific, with two main clusters being formed, one by *C. jejuni* strains and the other by *C. coli* strains (data not shown). In only one case did a human *C. coli* isolate have a *gyrA* sequence matching that of *C. jejuni*.

Other frequently found mutations in *gyrA* were (positions in our sequences given in parentheses): G118T (G11T), T234C (T127C), C243T (C136T), and C330T (C223T) in *C. jejuni*; C252T (C145T) and T297C (T190C) in *C. coli*; and T117C (T10C) in isolates of both species. None of these mutations

TABLE 2. Summary of MLST data, as well as source and number of isolates from Switzerland analyzed

| Species | Clonal complex[a] | ST[b] | No. from: | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Human | Poultry | Pig | Cattle | Dog | Cat | Water | Unknown |
| *C. jejuni* | 21 | 19 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | 21 | 4 | 8 | 0 | 3 | 3 | 1 | 0 | 0 |
| | | 47 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| | | 50 | 4 | 4 | 0 | 1 | 0 | 1 | 0 | 0 |
| | | 53 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | 141 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| | | 262 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | 451 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | 883 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| | | 917 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 |
| | | 1519 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| | 22 | 22 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 0 |
| | | **3361** | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| | 42 | 42 | 0 | 3 | 0 | 0 | 1 | 0 | 0 | 0 |
| | 45 | 11 | 0 | 3 | 0 | 0 | 0 | 1 | 0 | 0 |
| | | 44 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | 45 | 3 | 9 | 0 | 2 | 2 | 0 | 0 | 0 |
| | | 137 | 0 | 0 | 0 | 0 | 2 | 1 | 0 | 0 |
| | | 230 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | 233 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | 538 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 48 | 48 | 3 | 4 | 0 | 1 | 1 | 2 | 0 | 0 |
| | | 475 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| | | **3334** | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 49 | 49 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 52 | 52 | 3 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| | | 775 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| | 61 | 61 | 1 | 0 | 0 | 2 | 0 | 0 | 0 | 0 |
| | 206 | 46 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | 122 | 1 | 2 | 0 | 1 | 2 | 1 | 0 | 0 |
| | | 227 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | 572 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 |
| | | **3335** | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| | 257 | 257 | 2 | 1 | 1 | 1 | 0 | 0 | 0 | 0 |
| | | 367 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | 584 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | 824 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 283 | 564 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | **3362** | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 353 | 82 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | 353 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 |
| | | **3327** | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | 356 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 354 | 354 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | 878 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| | | 1476 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | 2288 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| | | **3325** | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 433 | **3329** | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 443 | 51 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 0 |
| | | 443 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | 2034 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 446 | 2899 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 460 | 606 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | **3333** | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 607 | 607 | 0 | 1 | 0 | 1 | 2 | 0 | 0 | 0 |
| | | 2310 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | **3324** | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| | 658 | 658 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 677 | 677 | 2 | 1 | 0 | 0 | 0 | 2 | 0 | 0 |
| | | 794 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 |
| | ND | 464 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| | ND | 586 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| | ND | 1035 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | ND | 1911 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |

*Continued on following page*

TABLE 2—*Continued*

| Species | Clonal complex[a] | ST[b] | No. from: | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Human | Poultry | Pig | Cattle | Dog | Cat | Water | Unknown |
| | ND | 2153 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| | ND | 2258 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| | ND | **3326** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| | ND | **3328** | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| | ND | **3330** | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | ND | **3331** | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | ND | **3332** | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *C. coli* | 828 | 825 | 3 | 1 | 2 | 0 | 1 | 0 | 0 | 0 |
| | | 828 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 |
| | | 854 | 0 | 9 | 38 | 0 | 0 | 0 | 0 | 0 |
| | | 901 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | 1016 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| | | 1096 | 1 | 1 | 4 | 0 | 0 | 0 | 0 | 0 |
| | | 1191 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| | | 1413 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 0 |
| | | 1556 | 0 | 4 | 3 | 0 | 0 | 0 | 0 | 0 |
| | | 1563 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| | | 1585 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | 2139 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| | | 2718 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| | | 2733 | 0 | 1 | 6 | 0 | 0 | 0 | 0 | 0 |
| | | **2916** | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | **2917** | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | 3020 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | 3023 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 |
| | | 3072 | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | **3336** | 0 | 1 | 17 | 0 | 0 | 0 | 0 | 0 |
| | | **3337** | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 |
| | | **3338** | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| | | **3339** | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | **3340** | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | **3341** | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| | | **3342** | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| | | **3343** | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 |
| | | **3346** | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| | | **3347** | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| | | **3349** | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 |
| | ND | 1009 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 |
| | ND | 1147 | 0 | 3 | 2 | 0 | 0 | 0 | 0 | 0 |
| | ND | 1426 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 |
| | ND | **2914** | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| | ND | **2915** | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| | ND | **2918** | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | ND | **3344** | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 |
| | ND | **3345** | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| | ND | **3348** | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Total | | | 76 | 77 | 100 | 23 | 24 | 17 | 1 | 3 |

[a] ND, not defined.
[b] New allele numbers are indicated in boldface.

were shown to be related to quinolone resistance as assessed phenotypically by MIC assays.

There was no relation between particular STs and the detected resistances.

**General sequence-based strain identification.** Genetic identification and phylogenetic investigation of the *C. jejuni* and *C. coli* strains were performed by sequence analysis of the 16S rRNA and *rpoB* genes. Phylogenetic analysis of the 16S rRNA gene fragments of all 186 *C. jejuni* and 143 *C. coli* strains confirmed previous studies showing that the resolution of this target is not high enough to separate these closely related species (10, 15). The 16S rRNA gene sequences of *C. jejuni* and *C. coli* match up to 100%. However, for the *rpoB* gene-based cluster analysis, *C. jejuni* and *C. coli* form separate groups, and differentiation between the species was possible. Moreover, two strains previously identified as *C. coli* were found to be *C. jejuni* by *rpoB* gene analysis. This could be further confirmed by MLST, since they belonged to ST3326 and ST257, respectively, which are from *C. jejuni*.

*C. jejuni* strains were grouped in four main clusters, I to IV, and *C. coli* isolates formed clusters V and VI (Fig. 3). Each of the four *C. jejuni* clusters corresponded to a type of *rpoB* sequence found in publicly available reference strains. Cluster I was closely related to *C. jejuni* NCTC 11351$^T$ (AF372097),

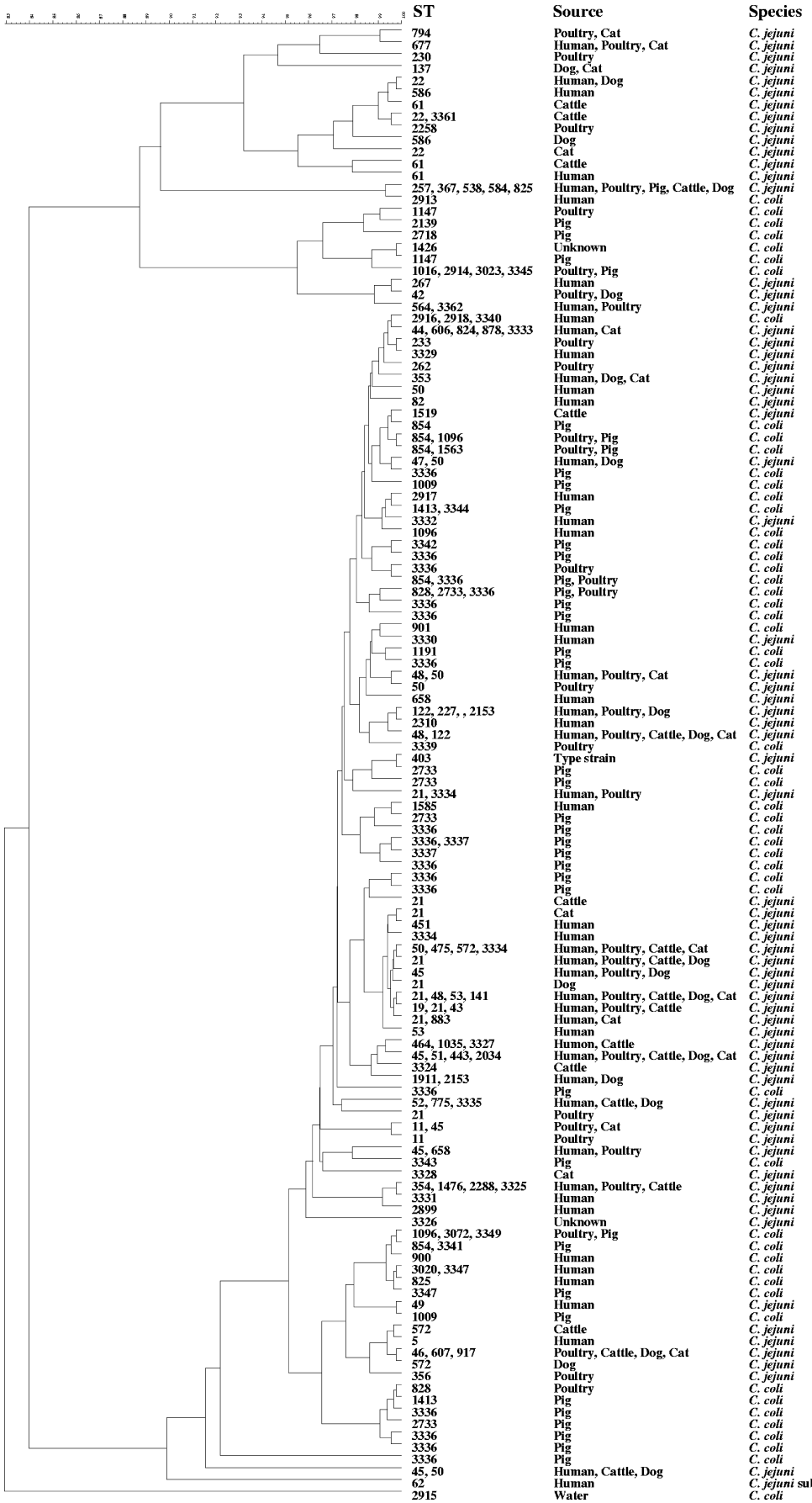


FIG. 1. Unweighted-pair group method using average linkages tree of the *flaA* gene fragment. The ST, source, and species are given.

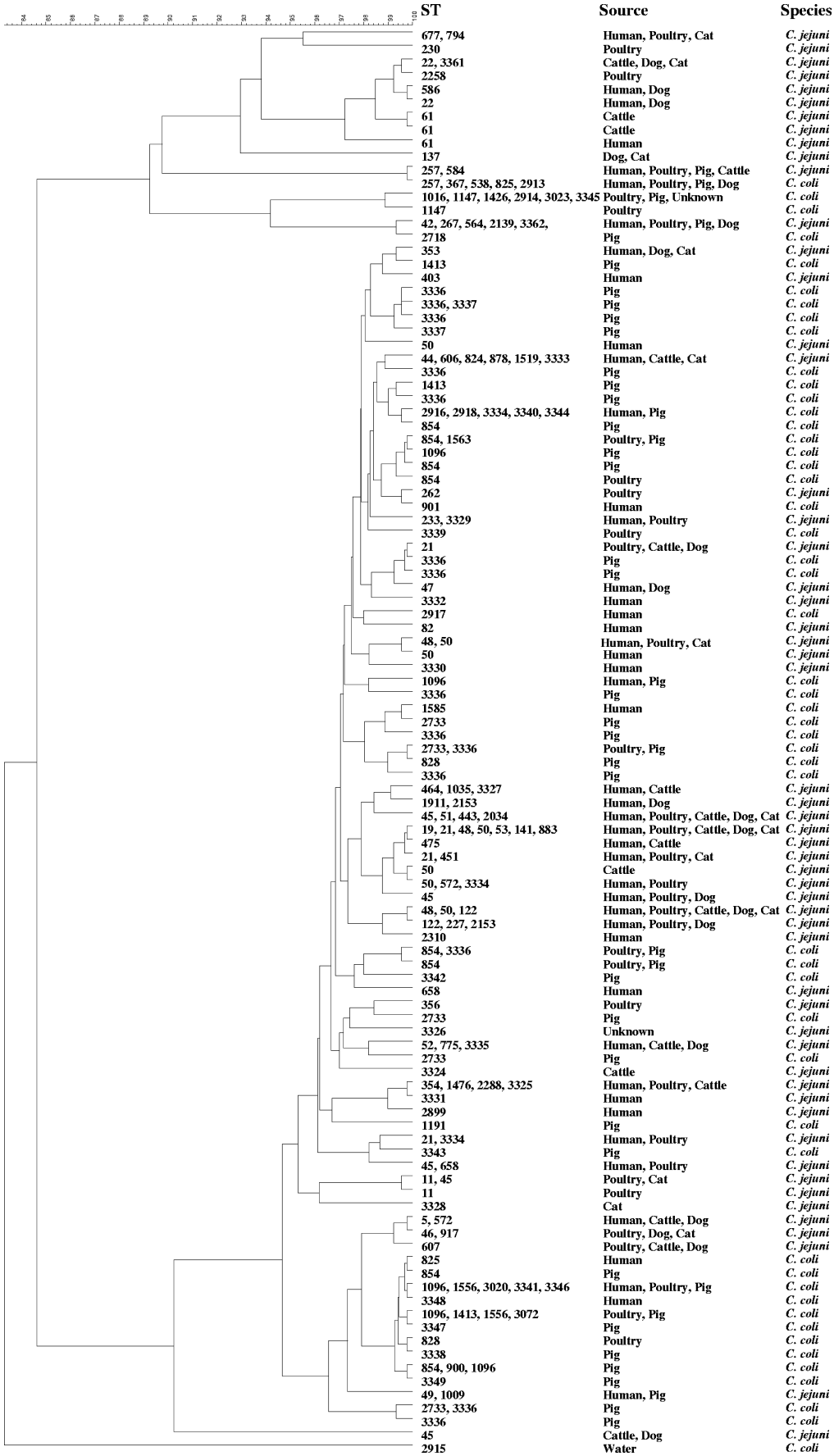


FIG. 2. Unweighted-pair group method using average linkages tree of the *flaB* gene fragment. The ST, source, and species are given.

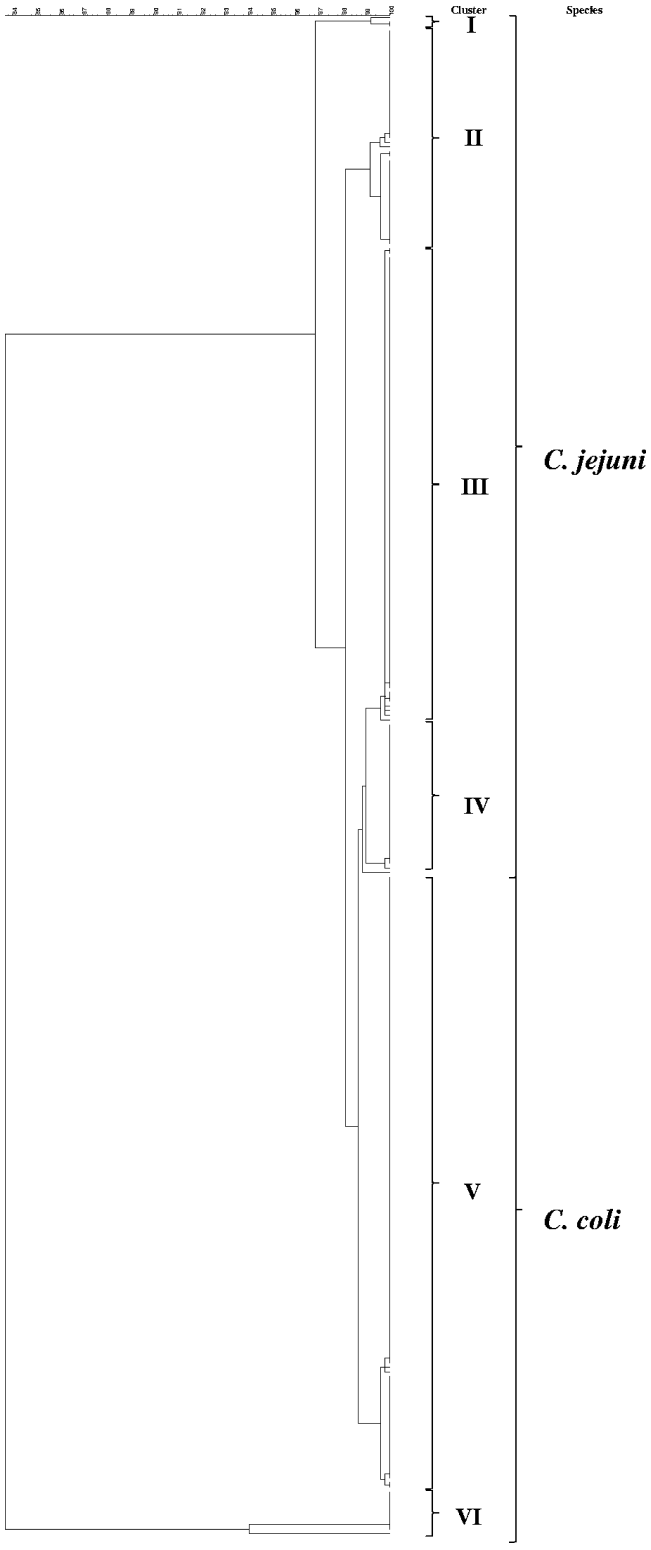


FIG. 3. Cluster analysis of *C. jejuni* and *C. coli* strains done in BioNumerics using the unweighted-pair group method using average linkages based on *rpoB* gene fragment sequences. The cluster number and species are given. The scale indicates percent sequence identity.

cluster II to *C. jejuni* 81-176 (CP000538), cluster III to *C. jejuni* NCTC 11168 (AL111168), and cluster IV to *C. jejuni* CCUG 12066 (DQ174200). Most *C. coli* isolates (93%) showed very high *ropB* sequence similarity (the highest sequence difference was 1 base out of 487) to the type strain of *C. coli* LMG 6440$^T$ (AF372098) and formed cluster V. A very distinct cluster, VI, was found for some *C. coli* isolates that were observed earlier (15). This group is mainly formed by multiple isolates of the previously known ST1147 and ST1426, as well as single isolates of the newly determined ST2914, ST2915, and ST3345.

In neither the 16S rRNA nor the *rpoB* gene tree was a correlation between clustering and the source of isolation observed.

## DISCUSSION

A new multiplex PCR and sequencing approach is presented as a modular, three-level genetic characterization system for *C. jejuni* and *C. coli*. This approach covers general identification, typing, and determination of antibiotic resistance. Previously established 16S rRNA and *rpoB* gene sequencing was applied for clear-cut identification of isolates. The MLST scheme was optimized to comprise a single and universal primer set for both species. Optimized primers were also designed for typing based on *flaA* and *flaB* genes, which can be included as an additional method and can be used alone or in combination with MLST. Finally, determination of macrolide and quinolone resistances was achieved by 23S rRNA and *gyrA* gene sequencing, respectively.

In order to minimize and optimize both handling and reagents, a multiplex PCR was set up, combining targets that differ in size, so that they yield specific products that are all amplified with equal efficiency. This resulted in the optimal combination of 12 targets in three AGs, thereby achieving a fourfold reduction in the number of PCRs and an optional single PCR for the 16S rRNA gene. The number of targets and resulting AGs described are exhaustive, and certainly not all 13 targets will be used for genetic characterization. Moreover, interests of laboratories in the various modules might be different. The reason for including so many targets was both to show proof of principle for the multiplex approach and to assess the usefulness of the individual modules. While the method was being evaluated, several target genes were combined, and the most promising have been chosen for the study of the *C. jejuni* and *C. coli* strain set collected in Switzerland.

In our experience, a purification step for PCR products is necessary to obtain high-quality sequences, which cannot be achieved if residual primers and other components of the PCR remain during the sequencing reaction. Column purification is normally used, but this method is expensive and inconvenient for high numbers of samples. An enzymatic purification step proved highly suitable and resulted in a significant improvement in sequence quality compared to nonpurified sample results, thus becoming a prerequisite for easy and efficient routine sequence analysis. Previously prepared sequencing plates containing the appropriate primers contributed to optimal handling during the preparation of sequencing reactions, and these plates can be stored at −20°C until they are used and are stable for at least several months. The format can be simply adapted to strips or single tubes, depending on the combina-

tion of targets and laboratory needs. Direct purification of sequencing reaction mixtures by a simple single-step ethanol precipitation is possible, and afterwards, the plates, strips, or tubes can be directly loaded on an automated sequencer without further transfer to new tubes.

A large collection of *C. jejuni* and *C. coli* strains from various sources were analyzed by the newly developed multiplex approach, which proved highly suitable, especially for MLST. High-quality, unambiguous sequence data could be generated by this procedure. The sequences of the various MLST target genes can be used in the assignment of classical STs after editing, or the full-length sequences can be used for further phylogenetic analysis using the appropriate software. Whereas STs provide easily comparable results for epidemiological purposes, phylogenetic analysis clearly shows the genetic relationships between isolates and thus also allows the separation of the two species. Moreover, while not all of the polymorphic sites located in the additional sequence protruding from the MLST target sequence segments used for typing by PubMLST influence the ST, they might allow further separation of strains belonging to the same ST, thereby increasing the resolution of the method.

The analyzed strain set represented a highly variable group of isolates, which is certainly based on the absence of epidemiological relationship of samples. Nonetheless, this study provides for the first time an overview of the various STs that can be found in Switzerland. The predominant CCs for *C. jejuni* were CC21 and CC45, which is the case in other European countries, indicating the wide distribution of these types (4, 6, 7, 13, 19, 33). Strains representing STs of both CCs were found in human and various animal species, except pigs. For *C. coli*, the greatest number of isolates were from ST845, which is found mainly in pigs, but also in poultry, and the newly determined ST3336, which is also isolated in both animal species. Interestingly, there was a relatively high number of STs detected and described for the first time, indicating that specific types are present in Switzerland that have not yet been found in other countries. More systematic studies with defined sample sets would help clarify this situation. Moreover, continuous probing and sampling of potentially contaminated food products, especially chicken, over a defined period of time and comparison of the STs detected with those isolated from human cases would lead to information about the potential risk of infection and provide data for intervention and prevention measures.

The typing of strains based on either *flaA* or *flaB* gave nearly overlapping results, which was also reflected in the 98.5% congruence between the two methods. The *flaA* gene showed higher discriminatory power than the *flaB* gene. However, in combination with MLST, *flaB* showed a slightly higher discriminatory ability than *flaA*. Moreover, the amplification of *flaA* was especially problematic in *C. coli* strains when the multiplex approach was used, and only the application of a specially designed optional forward primer for the amplification and sequencing of *flaA* solved the problem and resulted in high-quality sequences. However, this solution was not suitable for the multiplex approach. Since *flaB* was more stably amplified (99.4% of all strains) and is less prone to recombination than *flaA* (21), the former might be more suitable for typing, especially in combination with MLST.

The *fla* genes clustered strains in a very different way than MLST, and the isolates were distributed independently of their STs (Fig. 1 and 2), reflecting the fact that the two are different typing approaches and cannot be directly compared. This is also indicated by the absence of congruence between the two typing methods. In combination with MLST, the *fla* genes increased the discriminatory power of the method, which could be helpful in certain situations.

The proper identification of *Campylobacter* requires experience and might be difficult. Moreover, *Campylobacter* species other than *C. jejuni* and *C. coli* can be isolated from food poisoning and enteritis patients. In such cases, identification based on genetic markers, e.g., 16S rRNA and *rpoB* genes, might be helpful (15). The resolution of *rpoB* was higher than that of the 16S rRNA gene and even allowed separation between *C. jejuni* and *C. coli* (Fig. 3). Therefore, the *rpoB* gene might be fully sufficient for the identification of *Campylobacter* species, whereas the 16S rRNA gene might be helpful in identifying closely related and sometimes confounded species, such as those from the genus *Arcobacter* or *Helicobacter*.

*Campylobacter* evolves rather rapidly (31), and intra- and intergenomic changes not only occur in the environment, but also as a consequence of storage, culture, and passage in vitro. This might result in changes in the nucleotide sequences of different genes and should be taken into consideration when typing strains that have been subcultured over significant amounts of time (9, 11, 25, 35, 36). We have addressed this question by analyzing sequences of highly passaged strains with their progenitor. We found that the genes used for MLST and *fla* typing remained unchanged after more than 200 generations of in vitro subcultivation and are thus well suited for epidemiological investigation, an aspect that has not yet been addressed.

Both gene targets used for the genetic determination of antibiotic resistance to macrolides and quinolones could be efficiently sequenced by the multiplex approach. Moreover, mutations described in the literature as conferring antibiotic resistance were in all cases confirmed by the phenotypic MIC assays. None of the other observed additional mutations were associated with phenotypic resistance. Therefore, the included module for the genetic determination of antibiotic resistance is a highly valuable tool for the analysis of *C. jejuni* and *C. coli*. Analysis of isolates collected in Switzerland showed that none of the *C. jejuni* isolates were resistant to macrolides, whereas almost 21% of *C. coli* strains showed resistance against this group of antibiotics. With quinolones, 31% of *C. jejuni* and 40% of *C. coli* isolates were resistant. Finally, 6% of *C. coli* strains showed resistance to both classes of antibiotics. This reflects the fact that *C. jejuni* is predominantly found in poultry, whereas *C. coli* is mainly isolated from pigs, and antibiotic treatments used with the two animal species are different. The presence of antibiotic resistances demands the prudent use of these antibiotics in animal farming, especially in poultry and pig production.

Interestingly, one human isolate, which was clearly identified as *C. coli*, had a quinolone-resistant defining *gyrA* gene variant usually found in *C. jejuni* strains. This might be the result of recombination between the two species, a phenomenon which they are well known for (31, 39).

To improve and facilitate *Campylobacter* genotyping, not

only on the experimental level, but also on the analytical level, a combined *C. jejuni* and *C. coli* Web-based IDNS application service has been developed and made available by SmartGene. Analogous to other usages recently described (32), this platform allows the import of trace files from sequencers, editing, and proofreading by the integrated Proofreader, as well as straight allele, ST, and CC determination over an automated link/submission to the PubMLST database. In order to respond to questions related to epidemiology, the information on strains, their sequences, and final typing results are stored and can be cross-compared. Moreover, to facilitate multicenter collaborations, the software supports online networking between laboratories. While access to this system is protected, the Web technology allows laboratories to be easily connected so that they may access and share their data.

In summary, the MLST scheme for *C. jejuni* and *C. coli* was generalized, improved, and automated by establishing a multiplex approach. The approach was successfully applied in its most comprehensive form, including 13 target genes, to more than 300 *C. jejuni* and *C. coli* strains, yielding new information on types and antibiotic resistances of strains in Switzerland. Many laboratory-specific adaptations to the format (plates, strips, or tubes), as well as to the actual need (identification, MLST, *fla* typing, antibiotic resistance status, and their combinations) are possible. An IDNS platform allows easy and straightforward typing of isolates, as well as epidemiological analysis and strain tracing. The described approach contributes to accurate cost- and time-efficient monitoring and tracing of strains and to the development of effective prevention and intervention measures for *Campylobacter* infection.

## ACKNOWLEDGMENTS

This work was supported by a Swiss Federal Veterinary Office grant (1.06.04) and by the European Union-funded Integrated Project BIOTRACER (contract 036272) under the 6th RTD Framework.

We thank Vincent Perreten for his advice concerning antibiotic resistances in *Campylobacter* and MIC procedure.

## REFERENCES

1. **Bachoual, R., S. Ouabdesselam, F. Mory, C. Lascols, C. J. Soussy, and J. Tankovic.** 2001. Single or double mutational alterations of *gyrA* associated with fluoroquinolone resistance in *Campylobacter jejuni* and *Campylobacter coli*. Microb. Drug Resist. **7:**257–261.
2. **Clark, C. G., L. Bryden, W. R. Cuff, P. L. Johnson, F. Jamieson, B. Ciebin, and G. Wang.** 2005. Use of the Oxford multilocus sequence typing protocol and sequencing of the flagellin short variable region to characterize isolates from a large outbreak of waterborne *Campylobacter* sp. strains in Walkerton, Ontario, Canada. J. Clin. Microbiol. **43:**2080–2091.
3. **Clinical and Laboratory Standards Institute.** 2008. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals, 3rd ed., vol. 28, no. 8. Approved standard M31-A3. CLSI, Wayne, PA.
4. **Colles, F. M., K. Jones, R. M. Harding, and M. C. Maiden.** 2003. Genetic diversity of *Campylobacter jejuni* isolates from farm animals and the farm environment. Appl. Environ. Microbiol. **69:**7409–7413.
5. **Dingle, K. E., F. M. Colles, D. Falush, and M. C. J. Maiden.** 2005. Sequence typing and comparison of population biology of *Campylobacter coli* and *Campylobacter jejuni*. J. Clin. Microbiol. **43:**340–347.
6. **Dingle, K. E., F. M. Colles, D. R. Wareing, R. Ure, A. J. Fox, F. E. Bolton, H. J. Bootsma, R. J. Willems, R. Urwin, and M. C. J. Maiden.** 2001. Multilocus sequence typing system for *Campylobacter jejuni*. J. Clin. Microbiol. **39:**14–23.
7. **Duim, B., P. C. Godschalk, N. van den Braak, K. E. Dingle, J. R. Dijkstra, E. Leyde, J. van der Plas, F. M. Colles, H. P. Endtz, J. A. Wagenaar, M. C. Maiden, and A. van Belkum.** 2003. Molecular evidence for dissemination of unique *Campylobacter jejuni* clones in Curacao, Netherlands Antilles. J. Clin. Microbiol. **41**:5593–5597.
8. **European Food Safety Authority.** 2006. The community summary report on trends and sources of zoonoses, zoonotic agents, antimicrobial resistance and foodborne outbreaks in the European Union in 2005. Eur. Food Saf. Assoc. J. **94**:1–236.
9. **Gaynor, E. C., S. Cawthraw, G. Manning, J. K. MacKichan, S. Falkow, and D. G. Newell.** 2004. The genome-sequenced variant of *Campylobacter jejuni* NCTC 11168 and the original clonal clinical isolate differ markedly in colonization, gene expression, and virulence-associated phenotypes. J. Bacteriol. **186:**503–517.
10. **Gorkiewicz, G., G. Feierl, C. Schober, F. Dieber, J. Kofer, R. Zechner, and E. L. Zechner.** 2003. Species-specific identification of *Campylobacters* by partial 16S rRNA gene sequencing. J. Clin. Microbiol. **41:**2537–2546.
11. **Harrington, C. S., F. M. Thomson-Carter, and P. E. Carter.** 1997. Evidence for recombination in the flagellin locus of *Campylobacter jejuni*: implications for the flagellin gene typing scheme. J. Clin. Microbiol. **35:**2386–2392.
12. **Hunter, P. R., and M. A. Gaston.** 1988. Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. J. Clin. Microbiol. **26:**2465–2466.
13. **Kärenlampi, R., H. Rautelin, D. Schonberg-Norio, L. Paulin, and M. L. Hanninen.** 2007. Longitudinal study of Finnish *Campylobacter jejuni* and *C. coli* isolates from humans, using multilocus sequence typing, including comparison with epidemiological data and isolates from poultry and cattle. Appl. Environ. Microbiol. **73:**148–155.
14. **Keller, J., and V. Perreten.** 2006. Genetic diversity in fluoroquinolone and macrolide-resistant *Campylobacter coli* from pigs. Vet. Microbiol. **113:**103–108.
15. **Korczak, B. M., R. Stieber, S. Emler, A. P. Burnens, J. Frey, and P. Kuhnert.** 2006. Genetic relatedness within the genus *Campylobacter* inferred from *rpoB* sequences. Int. J. Syst. Evol. Microbiol. **56:**937–945.
16. **Kuhnert, P., J. Frey, N. P. Lang, and L. Mayfield.** 2002. A phylogenetic analysis of *Prevotella nigrescens*, *Prevotella intermedia* and *Porphyromonas gingivalis* field strains reveals a clear species clustering. Int. J. Syst. Evol. Microbiol. **52:**1391–1395.
17. **Levesque, S., E. Frost, R. D. Arbeit, and S. Michaud.** 2008. Multilocus sequence typing of *Campylobacter jejuni* isolates from humans,chickens, raw milk, and environmental water in Quebec, Canada. J. Clin. Microbiol. **46:** 3404–3411.
18. **Maiden, M. C., J. A. Bygraves, E. Feil, G. Morelli, J. E. Russell, R. Urwin, Q. Zhang, J. Zhou, K. Zurth, D. A. Caugant, I. M. Feavers, M. Achtman, and B. G. Spratt.** 1998. Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Proc. Natl. Acad. Sci. USA **95:**3140–3145.
19. **Manning, G., C. G. Dowson, M. C. Bagnall, I. H. Ahmed, M. West, and D. G. Newell.** 2003. Multilocus sequence typing for comparison of veterinary and human isolates of *Campylobacter jejuni*. Appl. Environ. Microbiol. **69:**6370–6379.
20. **Meinersmann, R. J., L. O. Helsel, P. I. Fields, and K. L. Hiett.** 1997. Discrimination of *Campylobacter jejuni* isolates by *fla* gene sequencing. J. Clin. Microbiol. **35:**2810–2814.
21. **Mellmann, A., J. Mosters, E. Bartelt, P. Roggentin, A. Ammon, A. W. Friedrich, H. Karch, and D. Harmsen.** 2004. Sequence-based typing of *flaB* is a more stable screening tool than typing of *flaA* for monitoring of *Campylobacter* populations. J. Clin. Microbiol. **42:**4840–4842.
22. **Mickan, L., R. Doyle, M. Valcanis, K. E. Dingle, L. Unicomb, and J. Lanser.** 2007. Multilocus sequence typing of *Campylobacter jejuni* isolates from New South Wales, Australia. J. Appl. Microbiol. **102:**144–152.
23. **Miller, W. G., M. D. Englen, S. Kathariou, I. V. Wesley, G. Wang, L. Pittenger-Alley, R. M. Siletz, W. Muraoka, P. J. Fedorka-Cray, and R. E. Mandrell.** 2006. Identification of host-associated alleles by multilocus sequence typing of *Campylobacter coli* strains from food animals. Microbiology **152:**245–255.
24. **Moore, J. E., D. Corcoran, J. S. Dooley, S. Fanning, B. Lucey, M. Matsuda, D. A. McDowell, F. Mégraud, B. C. Millar, R. O'Mahony, L. O'Riordan, M. O'Rourke, J. R. Rao, P. J. Rooney, A. Sails, and P. Whyte.** 2005. *Campylobacter*. Vet. Res. **36:**351–382.
25. **Nuijten, P. J., A. J. van den Berg, I. Formentini, B. A. van der Zeijst, and A. A. Jacobs.** 2000. DNA rearrangements in the flagellin locus of an *flaA* mutant of *Campylobacter jejuni* during colonization of chicken ceca. Infect. Immun. **68:**7137–7140.
26. **Price, E. P., V. Thiruvenkataswamy, L. Mickan, L. Unicomb, R. E. Rios, F. Huygens, and P. M. Giffard.** 2006. Genotyping of *Campylobacter jejuni* using seven single-nucleotide polymorphisms in combination with *flaA* short variable region sequencing. J. Med. Microbiol. **55:**1061–1070.
27. **Regula, G., R. Stephan, J. Danuser, B. Bissig, U. Ledergerber, D. Lo Fo Wong, and K. D. Stärk.** 2003. Reduced antibiotic resistance to fluoroquinolones and streptomycin in 'animal-friendly' pig fattening farms in Switzerland. Vet. Rec. **152**:80–81.
28. **Sails, A. D., B. Swaminathan, and P. I. Fields.** 2003. Clonal complexes of *Campylobacter jejuni* identified by multilocus sequence typing correlate with strain associations identified by multilocus enzyme electrophoresis. J. Clin. Microbiol. **41:**4058–4067.
29. **Sails, A. D., B. Swaminathan, and P. I. Fields.** 2003. Utility of multilocus sequence typing as an epidemiological tool for investigation of outbreaks of

gastroenteritis caused by *Campylobacter jejuni*. J. Clin. Microbiol. **41:**4733–4739.
30. **Schouls, L. M., S. Reulen, B. Duim, J. A. Wagenaar, R. J. Willems, K. E. Dingle, F. M. Colles, and J. D. Van Embden.** 2003. Comparative genotyping of *Campylobacter jejuni* by amplified fragment length polymorphism, multilocus sequence typing, and short repeat sequencing: strain diversity, host range, and recombination. J. Clin. Microbiol. **41:**15–26.
31. **Sheppard, S. K., N. D. McCarthy, D. Falush, and M. C. Maiden.** 2008. Convergence of *Campylobacter* species: implications for bacterial evolution. Science **320:**237–239.
32. **Simmon, K. E., A. C. Croft, and C. A. Petti.** 2006. Application of SmartGene IDNS software to partial 16S rRNA gene sequences for a diverse group of bacteria in a clinical laboratory. J. Clin. Microbiol. **44:**4400–4406.
33. **Sopwith, W., A. Birtles, M. Matthews, A. Fox, S. Gee, M. Painter, M. Regan, Q. Syed, and E. Bolton.** 2006. *Campylobacter jejuni* multilocus sequence types in humans, northwest England, 2003–2004. Emerg. Infect. Dis. **12:**1500–1507.
34. **Vacher, S., A. Menard, E. Bernard, and F. Megraud.** 2003. PCR-restriction fragment length polymorphism analysis for detection of point mutations associated with macrolide resistance in *Campylobacter* spp. Antimicrob. Agents Chemother. **47:**1125–1128.
35. **Wassenaar, T. M., B. N. Fry, and B. A. M. Van der Zeijst.** 1995. Variation of the flagellin gene locus of *Campylobacter jejuni* by recombination and horizontal gene transfer. Microbiology **141:**95–101.
36. **Wassenaar, T. M., B. Geilhausen, and D. G. Newell.** 1998. Evidence of genomic instability in *Campylobacter jejuni* isolated from poultry. Appl. Environ. Microbiol. **64:**1816–1821.
37. **Wassenaar, T. M., and D. G. Newell.** 2000. Genotyping of *Campylobacter* spp. Appl. Environ. Microbiol. **66:**1–9.
38. **Wieland, B., G. Regula, J. Danuser, M. Wittwer, A. P. Burnens, T. M. Wassenaar, and K. D. Stark.** 2005. *Campylobacter* spp. in dogs and cats in Switzerland: risk factor analysis and molecular characterization with AFLP. J. Vet. Med. B **52:**183–189.
39. **Wilson, D. J., E. Gabriel, A. J. Leatherbarrow, J. Cheesbrough, S. Gee, E. Bolton, A. Fox, C. A. Hart, P. J. Diggle, and P. Fearnhead.** 2008. Rapid evolution and the importance of recombination to the gastro-enteric pathogen *Campylobacter jejuni*. Mol. Biol. Evol.
40. **Wittwer, M., J. Keller, T. M. Wassenaar, R. Stephan, D. Howald, G. Regula, and B. Bissig-Choisat.** 2005. Genetic diversity and antibiotic resistance patterns in a *Campylobacter* population isolated from poultry farms in Switzerland. Appl. Environ. Microbiol. **71:**2840–2847.
41. **Zhang, Q., and P. J. Plummer.** 2008. Mechanisms of antibiotic resistance in *Campylobacter*, p. 263–276. *In* I. Nachamkin, C. M. Szymanski, and K. Blaser (ed.), *Campylobacter*. ASM Press, Washington, DC.