Comparative Analysis of Human and Canine *Campylobacter upsaliensis* Isolates by Amplified Fragment Length Polymorphism[▽]

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Human ($n = 33$) and canine ($n = 53$) *Campylobacter upsaliensis* isolates from seven countries were genotyped by a new amplified fragment length polymorphism method. We observed 100% typeability and high overall diversity. The majority of human strains (23/33) clustered separately from canine strains, indicating that dogs may not be the main source of human infection.

Campylobacter upsaliensis is a cause of sporadic gastrointestinal disease in humans (13). The sources of human *C. upsaliensis* infections are not well described, but dogs and cats appear to be the only significant animal reservoirs (1). These pet animals are often healthy carriers, and more than 50% of individual dogs and cats can be colonized (7, 9). Dog ownership has been described as a risk factor for human campylobacteriosis (2, 10), and case reports have provided evidence of pet-borne zoonotic transmission of *C. upsaliensis* (5, 6). Studying the genetic similarity between human and animal isolates is useful for determining whether pathogens like *C. upsaliensis* can be transmitted between different host species. Amplified fragment length polymorphism (AFLP) is one of the tools available for this purpose, but so far, the usage of AFLP for *C. upsaliensis* has been limited by factors such as low reproducibility (3), low typeability (16), and lack of discriminatory power (11).

In this study, we developed a new AFLP method to study the genetic diversity of human and canine *C. upsaliensis* isolates. A total of 86 *C. upsaliensis* strains obtained between 1983 and 2006 were analyzed, including 33 human clinical strains from seven countries (Belgium, Denmark, Senegal, South Africa, Sweden, the United Kingdom, and the United States) and 53 canine strains from two countries (Denmark and Sweden). Strains were grown on 5% blood agar plates (48 to 72 h, 37°C) under microaerophilic conditions, followed by DNA extraction using a High Pure PCR template preparation kit (Roche Diagnostics). Three microliters of genomic DNA was digested at 37°C for 2 hours with 10 units of BspDI and 10 units of NheI (New England Biolabs) in 14 µl of restriction buffer (14). For ligation, 5 µl of restriction product was transferred to a new tube containing 1 unit T4 DNA ligase (GE Healthcare), 2 µl 10× T4 ligase buffer (supplied with the enzyme), 8 µl restriction buffer, 2 µl 20 µM double-stranded NheI adaptor, and 2 µl 20 µM double-stranded BspDI adaptor (Table 1). Double-stranded adaptors were assembled immediately prior to the ligation step by mixing equimolar amounts of corresponding adaptor oligonucleotides. The mixtures were heated at 65°C for 10 min and cooled for 10 min at room temperature. Ligation was carried out overnight at room temperature. PCR amplification was performed in a 50-µl reaction volume containing 2 µl ligation product diluted 1:10 in Milli-Q water, 200 µM of each of the four deoxynucleoside triphosphates, 1.5 U *Taq* DNA polymerase (GE Healthcare), 2.75 µM MgCl$_2$, 10 µM Tris-HCl (pH 9.0), 50 mM KCl, 0.2 µM NheI primer, and 0.2 µM BspDI primer (Table 1). PCR was carried out on a GeneAmp 9700 system (Perkin-Elmer). The thermal program included initial denaturation at 94°C for 4 min succeeded by 30 cycles of 94°C for 60 s, 56°C for 60 s, and 72°C for 90 s and a 10-min final extension step at 72°C. For detection of AFLP fragments, 2 µl of PCR product was mixed with 2 µl formamide, 0.5 µl GeneScan 500-ROX size standard (Applied Biosystems), and 1.5 µl loading buffer (supplied with the size standard). The mixture was heated at 90°C for 2 min, cooled on ice, and loaded in aliquots of 1 µl on a 5% denaturing polyacrylamide gel. AFLP fragments were separated and detected on an ABI 377 automated sequencer (Perkin-Elmer) according to the manufacturer's instructions. AFLP profiles were analyzed with BioNumerics v. 4.5 (Applied Maths), using the Pearson product-moment correlation coefficient and clustering by the unweighted-pair group method with arithmetic averages. Band position tolerance and the optimization coefficient were set to 1.0% and 0.25%, respectively.

TABLE 1. Adaptor and primer oligonucleotides used for AFLP reaction

Oligonucleotide	Sequence[a]
BspDI adaptor	5′-CAG GTA TCG CCG AGT TTA GAC A-3′ 5′-CGT GTC TAA ACT CGG CGA TAC-3′
NheI adaptor	5′-AGG TGG ATT GGC CGT ATT ATT C-3′ 5′-CTA GGA ATA ATA CGG CCA ATC CA-3′
BspDI primer	5′-GCC GAG TTT AGA CAC GAT-3′
NheI primer	FAM-5′-GGC CGT ATT ATT CCT AGC-3′

[a] FAM, 6-carboxyfluorescein.

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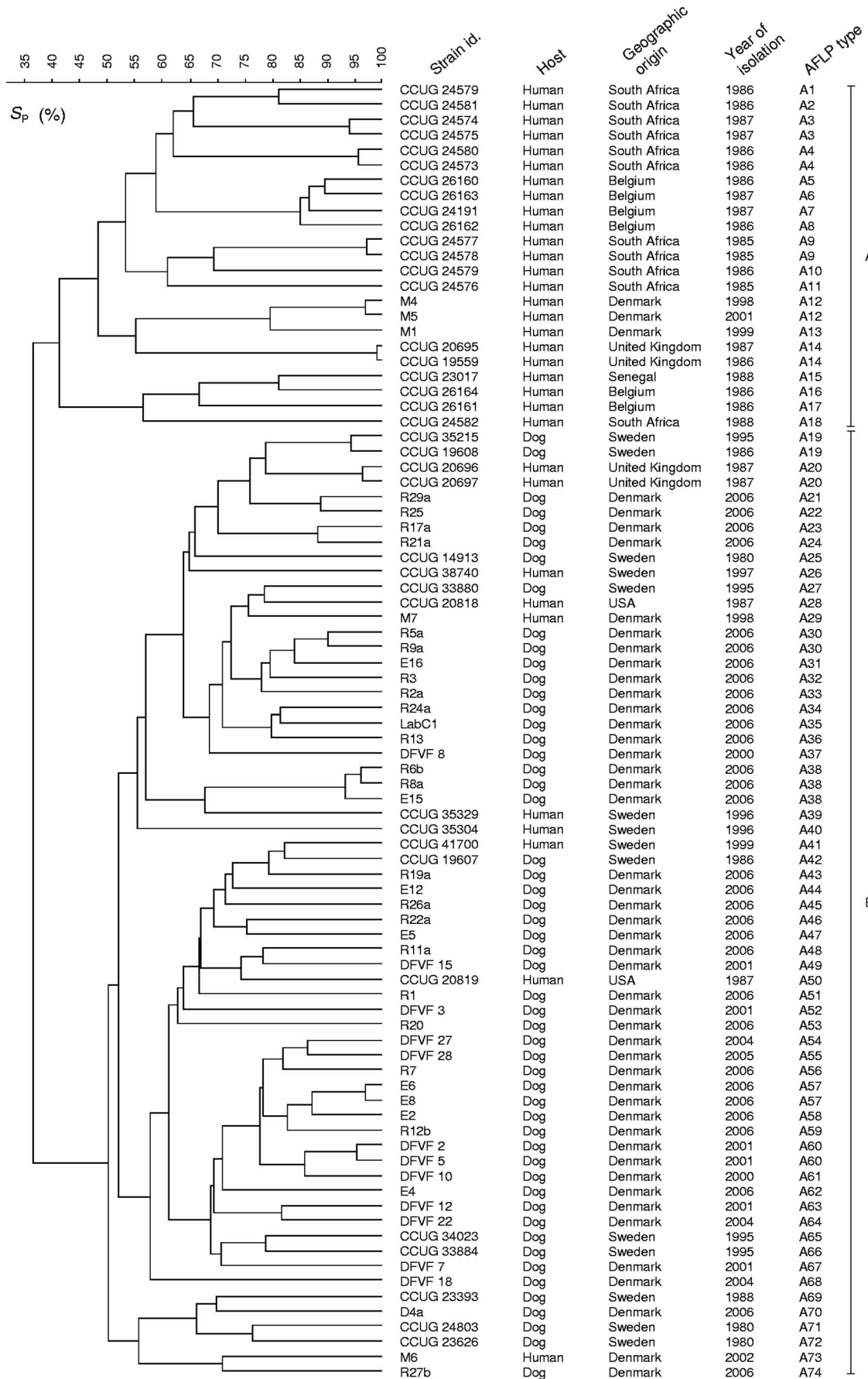
FIG. 1. Dendrogram showing genotypic similarities of 86 *C. upsaliensis* strains based on AFLP fingerprints. S_P, similarity calculated by the Pearson product-moment correlation coefficient.

All strains were typeable by AFLP analysis, and duplicate runs revealed a reproducibility of at least 90%. At the cutoff level of 90%, 74 different profiles were detected (Fig. 1). The AFLP fingerprints clustered into two main groups (A and B), sharing only 37% similarity (Fig. 1), thus confirming the high genetic heterogeneity typical for *Campylobacter* spp. (15). Group A was exclusively composed of human strains, whereas group B encompassed strains of both animal and human origins. This finding may be interpreted in various ways. First, *C. upsaliensis* subpopulations may be host specific, as previously suggested by Stanley et al. (12). However, the rare examples of healthy human carriers and the sporadic nature of *C. upsaliensis* infections dispute the existence and maintenance of such strains. Secondly, dogs may not be the main source of human infection and human strains belonging to group A might originate from cats or other yet undiscovered sources of *C. upsaliensis* infection. Finally, a biased sample population may have influenced the results. In fact, only human strains from Belgium, Senegal, South Africa, and the United Kingdom were analyzed and it cannot be excluded that canine strains from these countries would belong to group A.

The presence of certain indistinguishable profiles in group B gave insight into colonization and transmission dynamics among dogs. For example, the detection of indistinguishable AFLP patterns in strains isolated from dogs living within the same household (R17a and R21a) or rescue kennel (R25 and R29a) indicates that *C. upsaliensis* can be transmitted between dogs living in close contact (Fig. 1). Furthermore, it appears that dogs can be colonized with the same strain for relatively long periods, as indicated by the recovery of identical AFLP patterns in strains isolated from the same dog with a 1-month difference (DFVF 2 and DFVF 5) (Fig. 1). Some strains had indistinguishable AFLP profiles despite their being isolated several years apart. Among these were two Swedish dog strains (CCUG 19608 and CCUG 35215) from 1986 and 1995. These strains may represent stable clonal lineages, as previously suggested by other authors describing *Campylobacter* (8, 11).

In order to evaluate the typeability and discriminatory power of the AFLP protocol, a subset of 21 human and 21 canine strains were analyzed by a previously described pulsed-field gel electrophoresis (PFGE) method (7). Five strains showed no visible bands after two attempted runs, but results from the remaining 37 strains were in overall accordance with those obtained by AFLP. In some cases, AFLP was able to discriminate between strains sharing the same PFGE profile, and on one occasion, PFGE discriminated between two strains having the same AFLP profile (data not shown).

In conclusion, the AFLP method proved to be an easy, reproducible, and discriminatory tool for genotyping of *C. upsaliensis*. Spatial and temporal associations between epidemiologically related strains were detected, and the high genetic diversity of *C. upsaliensis* was confirmed. These results, combined with the perception that AFLP is less susceptible to genetic recombination than PFGE and other typing methods (4, 15), make this method suitable for both short- and long-term epidemiological studies of *C. upsaliensis*. The finding of a human cluster unrelated to canine strains emphasizes the need for more investigation to understand the epidemiology and population genetic structure of this bacterial species.

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