

Similarity of *Campylobacter lari* Among Human, Animal, and Water Isolates in Norway

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Abstract

A total of 49 isolates of *Campylobacter lari* from human, poultry, ducks, pigs, and water were genetically characterized. The species were identified by biotyping and multiplex polymerase chain reaction (PCR). Automatic riboprints were performed with the *Pst*I restriction enzyme and RiboPrinter[®]. The identification of the isolates was predicted when the corresponding pattern matched one of the patterns of the DuPont identification (DUP-ID) library and was then assigned an identification number. Thirty-five (71.4%) of the isolates were given a DUP-ID number. The isolates from water and animals showed a high degree of similarity to the human strains represented by DUP-PST1-1010, DUP-PST1-1166, DUP-PST1-1178, and DUP-PST1-1081. Some profiles (i.e., DUP-PST1-2021 and DUP-PST1-1184) were found only among the human isolates. Dendrogram analysis using BioNumerics grouped isolates into three main clusters. One of those clusters contained DUP-PST1-2021, DUP-PST1-1184, and DUP-PST1-1081, which was found in both humans and ducks. A second cluster generated DUP-PST1-1010, found in both humans and poultry, and DUP-PST1-1079, found in water. The third cluster consisted of two strains, DUP-PST1-1066 and DUP-PST1-1078, originating in humans, animals, and water. Three human strains and two poultry strains were diverse and formed their own clusters and could not be assigned a DUP-ID number. Because of the similarity of *C. lari* isolated from humans, poultry, ducks, pigs, and water, as well as the limited knowledge of environmental survival and its virulence factors, special hygienic precautions should be taken to avoid the risk of transmitting *Campylobacter*.

Introduction

THERMOPHILIC *CAMPYLOBACTER* spp., particularly *C. jejuni* and *C. coli*, are recognized as etiologic agents of acute diarrheal disease in humans worldwide (Skirrow, 1994; Nachamkin *et al.*, 1998). *Campylobacter* infections have surpassed salmonellosis as the most common water- or foodborne illness in many countries. In industrialized countries, *C. jejuni* and *C. coli* account for about 90% and 10% of human campylobacteriosis cases, respectively. In Norway, more than 2500 cases are diagnosed annually (MSIS-årsrapport 2005 and 2006). However, the

true rate of campylobacteriosis is estimated to be 10–100 times higher than reported (Kapperud, 1994).

Thermophilic *Campylobacter* represented by *C. jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis* are zoonotic pathogens with many animals serving as reservoirs for human disease. Reservoirs include rabbits, rodents, wild birds, sheep, horses, cows, pigs, poultry, and domestic pets (Rosef *et al.*, 1983; Kapperud and Rosef, 1983; Alterkruse *et al.*, 1994; Johnsen *et al.* 2006a). Contaminated vegetables and shellfish and cross-contamination with house flies may also be a source of infection (Rosef and Kapperud, 1983; Alterkruse

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et al., 1994; Ekdahl *et al.*, 2005; Nichols 2005). *C. lari*, associated with seabirds, is regularly isolated from gulls and puffins (Kapperud *et al.*, 1983; Moore *et al.*, 2002) and from the environment (Brown *et al.*, 2004; Johnsen *et al.*, 2006a). *C. lari*, however, has been reported to cause human disease less frequently than *C. jejuni* and *C. coli* (Martinot *et al.*, 2001). In Norway, approximately 3% of all isolates between 2001 and 2005 were identified as *C. lari* (Norwegian Institute of Public Health, personal communication).

Despite the high number of human cases of campylobacteriosis, knowledge of the pathogenesis and epidemiology of infection is still incomplete. The small number of identified outbreaks has typically been traced to contaminated milk or water (Friedmann *et al.*, 2000). The source of infection remains unidentified for most sporadic cases, although most case-control studies have indicated handling or consumption of raw or undercooked poultry as risk factors. Other risk factors consistently indicated include untreated water, raw milk, and contact with pets or farm animals (Friedman *et al.*, 2000).

Reliable and powerful typing methods are necessary in order to gain greater insight into the infection routes. Traditionally, phenotyping methods such as serotyping and biotyping have been used. The drawbacks of these methods are their restricted resolution, the lack of specific reagents for serotyping, and a large portion of untypeable strains. Genotyping to determine genetic relatedness is necessary to provide data for a better understanding of the epidemiological aspects of *Campylobacter* infections.

The purpose of this study was to characterize and compare *C. lari* isolated from human cases, poultry, ducks, pigs, and water by use of an automated *Pst*I ribotyping method to provide evidence of genetic relatedness among isolates. We used a standardized method coupled with a computer-based pattern analysis in order to compare ribotype profiles of *C. lari* in the DuPont identification (DUP-ID) library system.

Materials and Methods

Strain collection

Of 49 *C. lari* investigated, 18 human isolates and 21 poultry isolates were obtained from the

strain collection at the Norwegian Institute of Public Health collected through the NORM and NORM-Vet programs from 2001 to 2005 (Anonymous, 2005). Additionally, a strain from a pig, five water strains, and four strains from ducks were isolated in Telemark, Norway, in 2006.

Species identification

Biochemical differentiation. The isolates were kept frozen (-70°C) in MicrobankTM (Pro-Lab Diagnostics, Ontario, Canada) until further examination. The isolates were subcultivated and controlled for purity by phase contrast microscopy and by growth on nonselective blood agar plates. To distinguish among the species, the method described by Hwang and Ederer (1975) for detecting hydrolysis of hippurate was used. Susceptibility to nalidixic acid was evaluated on blood agar plates using antibiotic disks containing 30 μg of nalidixic acid (Oxoid Limited, Hampshire, England, UK).

Polymerase chain reaction (PCR) assay. The multiplex PCR primer sets described by Wang *et al.* (2002) were used for confirmation of the isolates. The colony multiplex PCR was optimized to simultaneously identify the 23S rRNA from *Campylobacter* spp. and the *glyA* gene (serine hydroxymethyltransferase) in *C. lari*. In short, a loopful of colony material from a single colony was suspended in 500 μL of water and centrifuged, and 5 μL of the supernatant was used as template in the PCR. The PCR mix consisted of the species-specific primers and multiplex master mix (Qiagen GmbH, Hilden, Germany), water, and template in a total volume of 50 μL . The PCR was run (iCycler, Bio-Rad Laboratories, Inc., Hercules, CA) with the following conditions: 95 $^{\circ}\text{C}$ for 6 minutes; 30 cycles of 95 $^{\circ}\text{C}$ for 30 seconds, 56 $^{\circ}\text{C}$ for 30 seconds, 72 $^{\circ}\text{C}$ for 30 seconds; 72 $^{\circ}\text{C}$ for 7 minutes; and finally ending at 4 $^{\circ}\text{C}$. The PCR products were run on a 1.5% agarose gel with Tris-acetate EDTA and ethidium bromide and visualized under UV light.

Ribotyping

Ribotyping was performed using the DuPont (Wilmington, DE) Qualicon RiboPrinter[®] as previously described (Bruce, 1996). Single colonies

from a 24-hour culture on blood agar plates were suspended in a sample buffer and heated at 80°C for 15 minutes. After the addition of lytic enzymes, samples were transferred to the RiboPrinter. Further analysis for the restriction of DNA, including use of the *Pst*I enzyme (20,000 U/mL, New England Biolabs, Inc., Ipswich, MA), was carried out automatically. The ribo-print profiles were aligned according to the position of a molecular size standard and compared with patterns stored in the library. The identification of an isolate was predicted when the corresponding patterns matched one of the patterns of the DUP-ID library of the RiboPrinter with a similarity > 0.85. The *Pst*I ribo-type patterns were automatically assigned a DUP-ID number (e.g., DUP-PST1-1010) by the RiboPrinter, which was confirmed by visual inspection. The profiles were transferred to and analyzed with the GelComparII software (Bio-Numerics, Applied Maths Inc., Gent, Belgium) using the Pearson correlation for genetic similarity and unweighted pair group method with average (UPGMA) clustering to determine profile relatedness.

Results

All 49 isolates were classified as *C. lari* according to biochemical tests and genotyping. Thirty-four isolates (71.4%) were given a DUP-ID number automatically from the RiboPrinter library as shown in Figure 1. The isolates were organized into three main clusters (Fig. 1). One of these contained the human clinical isolates DUP-PST1-2021, DUP-PST1-1184, and DUP-PST1-1081, which was found also in the duck isolates. The second cluster generated DUP-PST1-1010 found in both the human and poultry isolates, and a single strain DUP-PST1-1079 isolated from water. The third cluster consisted of DUP-PST1-1066 and DUP-PST1-1078 isolated from humans, animals, and water. Four human strains and two poultry strains were diverse and formed their own clusters and could not produce a DUP-ID.

Discussion

The natural habitat of thermophilic campylobacters is the intestinal tract of warm-blooded animals (Rosef *et al.*, 1983; Kapperud

and Rosef, 1983; Johnsen *et al.*, 2006a). Because of the high carriage rate of domestic and wild living animals, large numbers are excreted and provide a continuous flow into the environment. Besides the above-mentioned sources, human patients suffering from campylobacteriosis as well as healthy carriers also contribute to the flow of this organism into the environment. Although campylobacters do not seem to multiply outside their natural habitat, they may survive fairly well in the external environment, especially in aquatic niches (Blaser *et al.*, 1980; Steltzer *et al.*, 1991). Underestimating human cases due to cultivation methods that favor the isolation of *C. jejuni* and *C. coli* has been reported (Corry *et al.*, 1995). The maximum periods of viability of *Campylobacter* species at 4°C were found to be 3 weeks in feces, 4 weeks in water, and 5 weeks in urine (Steltzer *et al.*, 1991). Rosef *et al.* (1984) reported a survival of campylobacters on the surface of frozen poultry carcasses for several weeks. The habitat of *C. lari* is not fully understood, but it has been associated with gulls due to a high isolation rate and the salt tolerance of *C. lari* (Moore *et al.*, 2002). *C. lari* has caused waterborne outbreaks (Broczyk *et al.*, 1987). Because of the lack of the possibility for campylobacters to multiply in the environment, the epidemiological question is how long can they survive in the environment (including water) and cause infections. At present this is unknown.

Earlier serotyping of campylobacters isolated among domestic and wild animals (including birds) showed a high degree of diversity with 42 different serotypes among the typeable strains (65.7%) (Rosef *et al.*, 1985). Existence of tremendous genetic diversity within the *Campylobacter* species including *C. lari* has been documented (Meinersmann *et al.*, 2002). Broman *et al.* (2004) found a high degree of diversity among *C. jejuni* isolates from migrating birds with macro-restriction profiling (MRP) by pulsed-field gel electrophoresis (PFGE) using *Sma*I and *Kpn*I. A high degree of diversity was found from outdoor environment isolates (Johnsen *et al.*, 2006a) and among isolates from cattle (Johnsen *et al.*, 2006b) by using the restriction enzymes *Bgl*III and *Mfe*I and amplified fragment length polymorphisms (AFLPs). *C. lari* is a phenotypically and genotypically diverse organism. Duim *et al.*

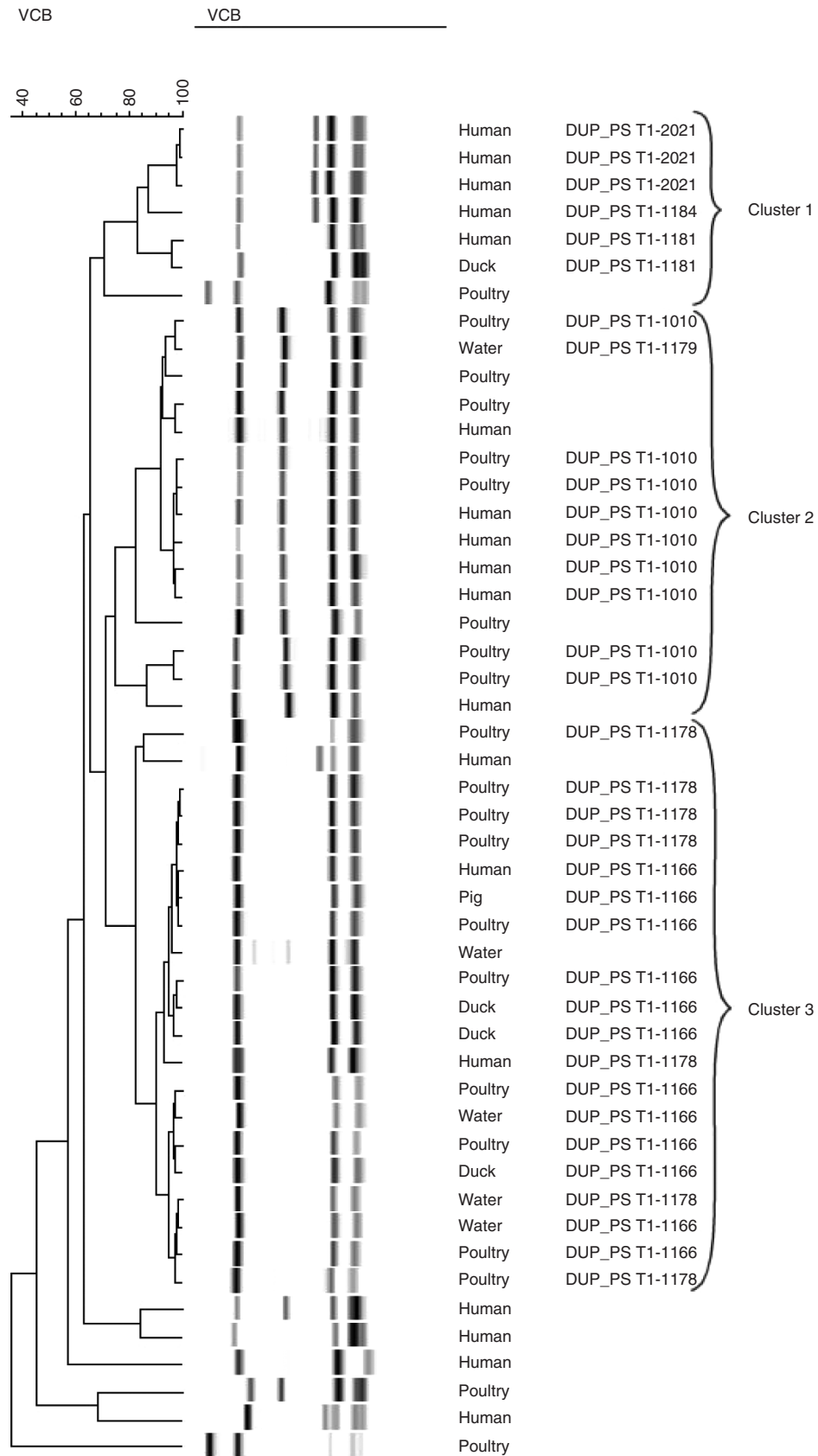


FIG. 1. Dendrogram of ribotype profiles of 49 *Campylobacter lari* strains.

(2004) found a great genetic heterogeneity in AFLPs using *Hind*III and *Hha*I restriction enzymes and protein profiles. Numerical analysis by AFLP profiles of *C. lari* is employable to study the relationship between the strains (Duim and Wagenaar, 2006). Ge *et al.* (2006) used both PFGE with the restriction enzyme *Sma*I and ribotyping with *Pst*I for genotyping and found genetic diversity among isolates from retail meats. Still, the knowledge of virulence and diversity of environmental strains including water is incomplete.

The restriction enzyme *Pst*I for the automatic ribotyping was chosen because of the library identification in the instrument and its use by other researchers (de Boer *et al.*, 2000). It is then possible to compare both the ribotypes with other users of RiboPrinter by the library, as well as ribotype profiles between the instruments. The population structure of thermophilic campylobacters has long been poorly understood. Results based on multilocus sequence typing showed a highly diverse organism (Dingle *et al.*, 2002). The organism's genetic instability can be said to have consequences for all methods of subtyping. Acquisition of foreign DNA and random recombination of large DNA segments may well cause alteration detectable using MRP by PFGE (Wassenaar *et al.*, 2000). Even so, MRP by PFGE has been used for the identification of infection sources and transmission routes during *C. jejuni* outbreaks (Hänninen *et al.*, 2003).

Cladistic parsimony analyses generated three clusters (Fig. 1). One cluster consists of DUP-PST1-1010, isolated from both poultry and humans, and DUP-PST1-1079, originating in water. Nineteen of the isolates given a DUP-ID number were located in the cluster consisting of DUP-PST1-1166 and DUP-PST1-1178. They seem to be common environmental strains from human, poultry, duck, pig, and water isolates and show a high degree of similarity. The DUP-PST1-2021 and DUP-PST1-1184 were found only among the human isolates and were assigned to the same cluster. This cluster generated DUP-PST1-1181, originating in both humans and poultry (Fig. 1). Four strains from humans and two from poultry show a high degree of variety and were generated in different clusters. They could not be assigned a DUP-ID number (Fig. 1).

Case-control studies have revealed non-disinfected drinking water and barbecuing as health hazards (Kapperud *et al.* 1992). Further, *C. lari* is regularly isolated from water (Rosef *et al.*, 2001; Hörman *et al.*, 2004), from pigs and birds (Rosef and Yndestad, 1982), and from outdoor environmental samples (Johnsen *et al.*, 2006b). We found a high degree of similarity between strains isolated from humans, poultry, ducks, pigs, and water (Fig. 1) by *Pst*I ribotyping. The interpretation of riboprint data in the dendrogram is based upon band position as well as signal intensity. Since the factors responsible for virulence in *C. lari* are unknown, it is quite possible that many environmental isolates may not be pathogenic, even though they belong to the same species and have identical ribotype profiles to clinical isolates. Due to the lack of detectable virulence factors of *C. lari* and the regular isolation from birds, other animals, and water, as well as its similarity to clinical human isolates, special precautions should be taken to avoid the risk of transmitting *Campylobacter*.

Conclusions

We used a highly standardized ribotyping method coupled with a computer-based pattern analysis in order to compare the strains. Use of the restriction enzyme *Pst*I makes comparing ribotype profiles of *C. lari* in the DUP-ID library system possible. The ribotypes DUP-PST1-1010, DUP-PST1-1166, DUP-PST1-1178, and DUP-PST1-1081 isolated from water and animals show a high degree of similarity to human clinical isolates.

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