DIVERSITY OF THERMOPHILIC *CAMPYLOBACTER* ISOLATED FROM THE BØ RIVER, SOUTHEAST NORWAY

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Summary. Out of 125 samples we isolated 45 thermophilic *Campylobacter* spp. from Bø River by filtration followed by growth on selective agar plates. The species were identified by biotyping, multiplex PCR and riboprinting. *C. jejuni* represented 17 (37.7%), *C. coli* 2 (4.4%), *C. lari* 5 (11.1%), *C. hyointestinalis* 1 (2.2%) and *C*. species 20 (44.5%). Five of *C. jejuni* were classified in ribogroups (DUP-PST1-ID) while four *C. lari* were identified in three. The RiboPrinter® identified *C. hyointestinalis*. Dendrogram analysis grouped the isolates into two main clades and seven subclades. *C. lari* and *C. coli* were grouped in one clade while most *C*. spp. and the *C. hyointestinalis* were grouped in the other. *C. jejuni* were clustered in five subclades and display a high degree of diversity. Because of the frequent isolation of campylobacters in surface water and the lack of knowledge of the pathogenesis of *Campylobacter* strains and their virulence factors, special hygienic precautions should be taken to avoid the risk of transmitting campylobacteriosis from water.

Key words: *Campylobacter*, diversity, water, clustering, genotyping, riboprinting.

TERMOFILINIŲ *CAMPYLOBACTER*, IŠSKIRTŲ IŠ BØ UPĖS PIETRYČIŲ NORVEGIJOJE, ĮVAIROVĖ

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Santrauka. Iš 125 Bø upės vandens filtravimo pavyzdžių, augintų ant selektyvios agaro terpės, buvo išskirti 45 termofilinių *Campylobacter* mėginiai. Rūšis nustatyta biotipuojant, dauginės PGR metodais ir automatinio identifikavimo prietaisu „RiboPrinter®“. *C. jejuni* sudarė 37,7 proc. (17 mėginių), *C. coli* – 4,4 proc. (2), *C. lari* – 11,1 proc. (5), *C. Hyointestinalis* – 2,2 proc. (1) ir neidentifikuotos rūšys *C. species* – 44,5 proc. (20). Penki iš 17 *C. jejuni* mėginių priklausė žinomiems ribogrupės DUP-PST1-ID bibliotekoje genotipams, o iš 4 *C. lari* priklausė trims genotipams. *C. hyointestinalis* nustatyti pagal žinomą „RiboPrinter®“ klasifikaciją. Genetinio panašumo analizės pavyzdžiai pasiskirstė į dvi sankaupas (klasterius) su septyniais subklasteriais. *C. Lari* ir *C. coli* priklauso vienai sankaupai, o dauguma *C*. spp. ir *C. hyointestinalis* – kitai. *C. jejuni* pasiskirstė į penkis labai įvairius subklasterius. Kadangi Bø upėje nustatyta daug kompilobakterijų, tarp kurių aptikti ir patogeniški štamai, būtinos specialios higienos apsaugos priemonės.

Raktažodžiai: *Campylobacter*, įvairovė, upės vanduo, genotipavimas, „RiboPrinter®“.

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/foodborne pathogen in many countries. In industrialized countries, cases of human campylobacteriosis due to *C. jejuni* and *C . coli* are about 90% and 10% respectively. In Norway, more than 2500 cases are diagnosed yearly (MSIS year-report 2005 and 2006). However, the true rate of campylobacteriosis is estimated to be 10-100 times higher than reported (Kapperud 1994). Campylobacters are frequently isolated from water and water supplies and have been the cause of infection in reported outbreaks in many countries including Norway (Mentzing 1981, Vogt et al. 1982, Rosef and Mork 1985, Dahl and Melby 1987, Melby et al. 1991, Koenraad et al. 1997).

Reliable and powerful typing methods for campylobacters are necessary in order to gain more insight into infection routes. Traditionally, phenotyping methods such as serotyping and biotyping have been used. The drawbacks of these methods are their restricted resolutions, the lack of specific reagents for serotyping, and a large portion of untypable strains. Genotyping to determine genetic relatedness is necessary to provide data for better understanding of the epidemiological aspects of the infections. The purpose of this study was to identify the thermophilic campylobacters isolated from river water to species level and use the automated *Pst*I ribotyping method to provide evidence of genetic relatedness.

Material and methods

Bacterial isolates

The water samples were collected into one litre sterile glass bottles from two localities in the Bø River in Telemark County, southeast Norway. Bø river is limited

influenced from agriculture and industrial contamination and is used for recreation and infiltration for drinking water. The samples (n=125) were collected from January to May 2006 and brought to the laboratory within two hours for immediate bacteriological analysis. The water temperature through the sampling period varied from 1.0-5.2 ºC. The water samples (100 mL) were filtered using a 0.45□m pore size filter (Millipore Corporate Headquarters, Billerica, MA). The filter was placed face up on modified blood-free CCDA-Preston agar (Oxoid CM 739 and SR 155) (Oxoid Limited, Hampshire, England). The plates were incubated at 42ºC under microaerobic conditions by using the Oxoid CampyGen in an anaerobic jar. The plates were read after 24 and 48 hours, and typical colonies were confirmed by phase contrast microscopy. The isolates were kept frozen (-70ºC) in Microbank™ (Pro-Lab Diagnostics, Canada) until further examinations. Forty-five isolates were collected for further characterization.

Species identification

Biochemical differentiation

The isolates were subcultivated and controlled for purity by phase contrast microscopy and by growth on non-selective 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 □g nalidixic acid (Oxoid Limited, Hampshire, England).

PCR-assay

The multiplex-PCR primer sets described by Wang et al. (2002) were used for species confirmation of the isolates. The colony multiplex-PCR was optimized to simultaneously identify the 23S rRNA from *C.* spp., the *hipO* gene from *C. jejuni*, and the *glyA* gene from *C. coli, C. lari* and *C. upsaliensis.* In short, a loopful of colony material from a single colony was suspended in 500µL of water, centrifuged, and 5 µL of the supernatant was used as template in the polymerase chain reaction (PCR). The PCR-mix consisted of the species-specific primers and multiplex master mix (Quiagen GmbH, Hilden, Germany), water and template in a total volume of 50µL. The PCR was run (iCycler, Bio-Rad Laboratories, Inc., Hercules, CA) with the following conditions: 95ºC for 6 minutes, 30 repetitions of 95ºC for 30 seconds, 56ºC for 30 seconds, 72ºC for 30 seconds and finally 72ºC for 7 minutes. The PCR products were run on a 1.5 % agarose gel with TAE and ethidium bromide and visualized under UV light. *C. coli, C. jejuni, C. lari* and *C. upsaliensis* strains were used as positive controls.

Riboprinting

Ribotyping was performed using the DuPont (Wilmington, DE) Qualicon RiboPrinter® as previously described (Bruce 1996). Single colonies from a 24-h culture on blood agar plates were suspended in sample buffer and heated at 80ºC for 15 min. 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 (20.000U/ML, New England Biolabs), was carried out automatically. The riboprint 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 pattern of the DuPont identification library of the RiboPrinter® with a similarity of > 0.85. The *Pst*I ribotype patterns were automatically assigned a DuPont identification number (e.g. DUP-PST1-1166) by the RiboPrinter®, which was confirmed by visual inspection. The profiles were transferred and analyzed with the GelComparII® software (Bio Numerics, Applied Maths Inc., Gent, Belgium) using the Pearson correlation and default settings for optimization (2.0%) and position tolerance (1.00%) for genetic similarity. The dendrogram was generated by the unweighted pair group method with arithmetic averages (UPGMA) to determine profile relatedness.

Results. Thermophilic campylobacters were isolated in 45 out of 125 samples (36%). The biochemical and genetic differentiation of the isolates into species level are shown in Table 1. *C.* spp. dominated with 20 isolates, followed by *C. jejuni* with 17, *C. lari* with 5, *C. coli* with 2 and *C. hyointestinalis* with one. *C.* spp. did not hydrolyse hippurate and were sensitive to nalidixic acid, but it could be classified as *C.* spp. by typical shape by phase contrast microscopy and specifically identifying 23S rRNA by PCR. Five DUP-PST1-IDs were found among the 17 *C. jejuni* and four of the five isolates of *C. lari* were represented by three ribotypes (Table 1). Among the most frequently isolated *C.* spp. only one *C. hyointestinales* was identified by the RiboPrinter® library and given a DUP-PST1-ID (Table 1). Cladistic parsimony analysis generates two main clades and seven subclades, as shown in Figure 1. One main clade generated four subclades consisting of *C. lari, C. coli* and some genotypes of *C. jejuni* and *C.* spp.: the first subclade consisted of some *C. jejuni* and *C.* spp., the second consisted of all *C. lari*; the third consisted of *C. jejuni* and one *C.* spp.; and the fourth included *C. jejuni* and *C. coli*. The other main clade generated three subclades, two of which consist of *C. jejuni* and *C.* spp., while the third consists of *C. hyointestinalis* and *C.* spp. *C. jejuni* represented a high diversity of *Pst*I ribotypes with15 different profiles, while the five *C. lari* isolates represented three profiles. The *C.* spp. also displays high diversity while the two isolates of *C. coli* were similar.

Discussion. There are no standard methods for detecting campylobacters in water. Rosef et al. (1987) demonstrated isolations of campylobacters by procedures based on membrane filtration followed by an enrichment procedure and direct plating of the filtrated membranes, and this comparative study has been followed up by Brennhovd and Kapperud (1991a). The degree of faecal contamination can be expressed by index bacteria. Multiple logistic modelling by using faecal coliforms as index bacteria for predicting the occurrence of *C. jejuni* and *C. coli* in water as a tool for risk analysis have been carried out by Skjerve and Brennhovd (1992).

Table 1. **Biochemical and genetic differentiation of 45 *Campylobacter* isolates from Bø river**

Species	Campylobacter multiplex PCR	Biotyping* Hippurate	Nalidixic acid	DUP-PSTI-Ribotype	%
C. jejuni	17	17	0	1122 1130 2000 2058	37.7
C. coli	2	0	0		4.4
C. lari	5	0	5	1166 (n=2) 1178 1179	11.1
C. upsaliensis	0	0	0		0
C. spp.	20	0	0		44.4
C. hyointestinalis	1	0	0	1170	2.2

* Hippurate hydrolysis and resistance to nalidixic acid

In an earlier study Rosef et al. (2001) isolated *C.* spp. in 53.3% of water samples in the Bø river. A high degree of contamination has also been reported in Finland (Hörman et al. 2004). The organisms, however, may remain dormant in water in a state that has been termed "viable but non-culturable" (Rollins and Colwell 1986), meaning that under unfavourable conditions, the organisms essentially remain dormant and cannot be easily recovered on artificial media.

A high frequency of thermophilic campylobacters was isolated, representing 45 out of 125 water samples (36%). In spite of the lack of optimal isolation procedures, campylobacters were isolated from surface water that must be characterized as heavily contaminated, which is in accordance with earlier studies (Rosef et al. 2001). The efficiency of any isolation procedure in recovering campylobacters from water depends on the quality of the water to be tested. Among the factors which influence recovery are the number of campylobacters present, turbidity, level of contamination, type of competing microorganisms, and volume of water examined (Thomas et al. 1999). Brennhovd and Kapperud (1991b) found the highest detection rate of campylobacters in water between 2º and 12ºC. The present study was carried out at water temperatures between 1.0º and 5.2ºC. Fewer competition factors may be present at lower temperatures, as evidenced by Gondrosen (1986), who found a survival for 15 days at 4ºC. Waterfowl are regular visitors to most surface water. Because of a higher body temperature, birds have different faecal flora than mammals and normally a lower content of faecal coliforms, but ones which are favourable for campylobacters. Two waterborne outbreaks of campylobacteriosis caused by geese (*Anser brachyrhynchus*) are described by Varslot et al. (1996) while an outbreak with *C. lari* is associated with gulls (Broczyk et al. 1987). It is likely that contamination from birds plays a major role in contamination and transmission routes into water. *C. jejuni, C. coli* and *C. lari* were all formed in one clade (Figure 1).These species are normal isolates among birds. (Kapperud and Rosef 1983, Waldenstrøm 2005).

The natural habitat of thermotolerant campylobacters is the intestinal tract of warm-blooded animals (Kapperud and Rosef 1983, Rosef et al. 1983, Alterkruse et al. 1994, Brown et al. 2004, Johnsen et al. 2006a). Because of the high carrier rate in 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 provide a flow of this organism into the environment. The dendrogram (Figure 1) illustrates that the other clade contains most of the *C.* spp., some *C. jejuni,* and the only *C. hyointestinalis* isolate. The source(s) of these is unknown.

Although thermophilic 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, Thomas et al. 1999). The maximum period of viability of *C.* spp. at 4ºC was found to be 3 weeks in faeces, 4 weeks in water and 5 weeks in urine (Steltzer et al. 1991). Rosef et al. (1984) found a survival of campylobacters on the surface of frozen poultry carcasses to be several weeks. Because of the lack of the possibility for campylobacters to multiply in the environment, the epidemiological question is how long they can survive in the environment (including water) and cause infections. At present this is unknown. However, because of the contamination of surface waters, exposure during bathing or other recreational activities may increase the probability of contracting campylobacteriosis. The infective dose is very low with only a few hundred bacteria necessary to cause human infection (Black et al. 1988). Children in particular may be at risk when swallowing contaminated water while bathing. The present results indicate that hygienic precautions should be taken when procuring drinking water from surface waters.

Earlier works on serotyping of *C.* spp. isolated among domestic and wild animals (including birds) displayed diversity with 42 different serotypes among the typeable strains (65,7%) (Rosef et al.1985). Broman et al. (2004) found a high degree of diversity among isolates from migrating birds with MRP (macrorestriction profiling) by

PFGE (pulsed-field gel electrophoresis) using *Sma*I and *Kpn*I, and only 2 out of 89 isolates tested were identical or very similar to isolates from humans. By using the restriction enzymes *Bgl*II and *Mfe*I and amplified-fragment length polymorphism (AFLP), Johnsen et al. (2006a) found a high degree of diversity among outdoor environmental isolates and isolates from cattle (2006b).

Knowledge of the diversity of the environmental strains from water is lacking. We used a highly standardized method coupled with a computer-based pattern analysis in order to compare the strains, which then provides the possibility to compare ribotype profiles and the strains in a dendrogram.

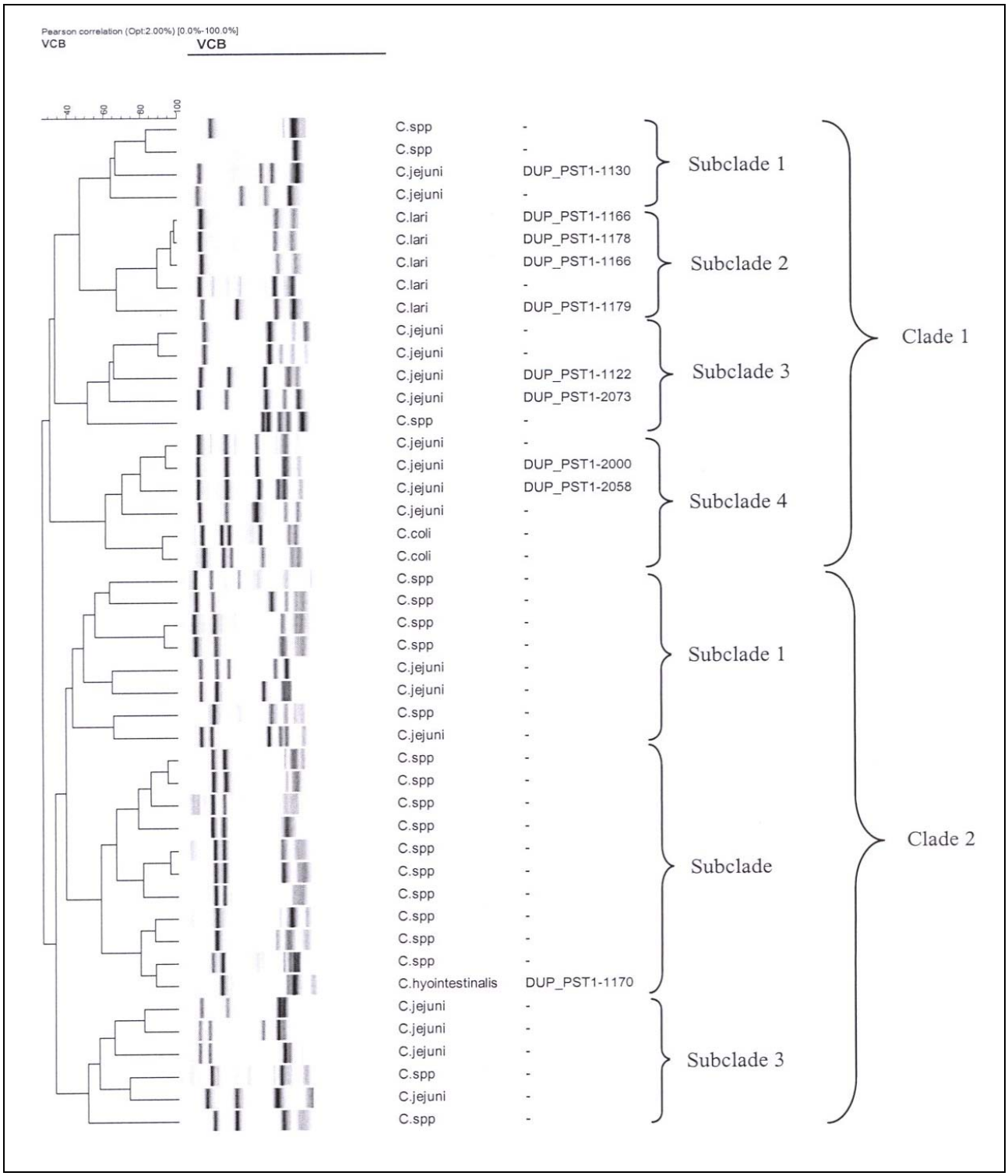

Figure 1. **Ribotype profiles of 45 thermphilic campylobacters isolated from Bø river**

The restriction enzyme *Pst*I for automatic ribotyping was used because of the Qualicon library identification (DUP-PST1-ID) in the RiboPrinter® and the use by other researchers (de Boer 2000). It is then possible to compare the ribotype pattern with other users of a RiboPrinter®. A number of different ribotypes were found between and within the species as shown in Figure 1. Out of *C.* spp. (n=21), only one could be given a DUP-ID and was classified as *C. hyointestinalis* (Table 1). *C. lari* could be clustered in three ribogroups where two are regularly found among human and poultry isolates (Rosef et al. 2008).

Automatic riboprinting to identify isolates from a waterborne outbreak has also been ruled out (Nielsen et al. 2000) while Hänninen et al. (2003) used MRP for the identification of infection sources and transmission routes during *C. jejuni* outbreaks from water. We identified 15 *C. jejuni* profiles (Figure 1). Water used for agricultural purposes may spread the bacteria to vegetables, though no data on the prevalence of campylobacters on vegetables is available. No standard method for isolation of the bacteria from the environment including vegetables exists, but new methods based on PCR for detecting the bacteria from environmental samples and foods have been established (Waage et al. 1999, Straub et al. 1999). Case-control studies, however, have revealed non-disinfected drinking water and barbecuing as health hazards (Kapperud et al. 1992).

The highest similarity to human clinical isolates has been observed in poultry isolates, while other birds and domestic animals have shown less similarity (Munroe et al. 1983, Rosef et al. 1985, Broman et al. 2004). Since the factors responsible for virulence are unknown, it is quite possible that many environmental isolates may not be pathogenic, even though they belong to the same species, are serologically identical and belong to same ribogroup as clinical isolates. Due to the lack of detectable virulence factors and the high number of isolates from surface water, special precautions should be taken to avoid the risk of transmitting campylobacteriosis by water.

Conclusion. The potential pathogenic thermophilic *C. jejuni, C. lari* and *C. coli* are regularly isolated from river water. Riboprints performed by the *Pst*I enzyme showed high diversity between the species and within the species. The RiboPrinter® cannot be used for species identification because too few isolates are characterized in the library. The isolates could be divided into two clades and seven suclades (UPGMA). The *Campylobacter* species in one of these is similar to those usually isolated from birds. The strains generating the other clade cannot give any information about the source. Because of the limited knowledge of the virulence factors and strains, special hygienic precautions should be taken to avoid the risk of transmitting campylobacteriosis by water.

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