

DIVERSITY OF THERMOPHILIC *Campylobacter* ISOLATED FROM SLAUGHTER PIGS IN NORWAY

Olav Rosef¹, Algimantas Paulauskas², Aud Stølan¹, Elisabeth Moen Bråthen¹, Camilla Haslekås¹

¹Telemark University College, Telemark, Norway,

²Vytautas Magnus University, Kaunas, Lithuania

Correspondence: Olav Rosef, Telemark University College, Hallvard Eikas Plass 1, 3800 Bø i Telemark, Norway

Fax: +47 35952703. E-mail: olav.rosef@hit.no, Telephone +47 35952782

Summary. Out of 100 faecal swab samples, 88 thermophilic *Campylobacter* representing ten herds were isolated. The species were identified by biotyping and multiplex polymerase chain reaction (PCR). Of the isolates, 86 (97.7%) were *C. coli*, one was *C. lari*, and one was *C. jejuni*. All three species were isolated from the same herd. Automatic riboprints were performed with the *PstI* restriction enzyme and RiboPrinter® (automated microbial characterization system). The identification of the isolates was predicted when the corresponding pattern matched one of the patterns in the DuPont identification (DUP-ID) library and was then assigned an identification number. A total of 81 selected *C. coli* isolates were riboprinted. The isolates could be divided into two clades and five subclades, and 19 isolates could be given five different DUP-IDs from the library: DUP-PST1-1182 (n=6), DUP-PST1-1208 (n=10), DUP-PST1-1175 (n=1), DUP-PST1-1140 (n=1), and DUP-PST1-1163 (n=1). Pigs are healthy carriers of *C. coli* which showed a high degree of diversity among and within the herds.

Keywords: *Campylobacter*, diversity, pig, clustering, genotyping, riboprinting.

TERMOFILINIŲ *Campylobacter* IZOLIATŲ IŠ KIAULIŲ SKERDIENOS ĮVAIROVĖ NORVEGIJOJE

Olav Rosef¹, Algimantas Paulauskas², Aud Stølan¹, Elisabeth Moen Bråthen¹, Camilla Haslekås¹

¹Telemark University College, Hallvard Eikas Plass 1, 3800 Bø i Telemark, Norway;

faks.: +47 3595 2703; tel. +47 3595 2782; el. paštas: olav.rosef@hit.no

²Vytauto Didžiojo universitetas, Vileikos g. 8, Kaunas, Lietuva

Santrauka. Iš 100 kiaulių fekalijų tepinėlių 88 rastos termofilinės *Campylobacter*. Rūšis nustatyta biotipuojant ir dauginės PGR metodais: 86 (97,7 proc.) pavyzdžiuose nustatyta *C. coli*, po vieną *C. lari* ir *C. jejuni*. Visos trys bakterijos rūšys išskirtos iš tos pačios bandos gyvulių. Automatinis genotipavimas atliktas naudojant restrikcijos fermentą *PstI* ir prietaisą „RiboPrinter®“. Tirtos bakterijos įvertintos lyginant su DuPont identifikavimo (DUP-ID) bibliotekos katalogo pavyzdžiais, joms suteiktas atitinkamas identifikacijos numeris. „RiboPrinter®“ prietaisu genotipavus 81 *C. coli* pavyzdį, atlikta klasterinė analizė parodė, kad tirti pavyzdžiai pasiskirsto į dvi sankaupas (klasterius) su penkiais subklasteriais. 19 pavyzdžių priklauso penkiems skirtingiems žinomiems DUP-ID bibliotekos katalogo genotipams: DUP-PST1-1182 (n=6), DUP-PST1-1208 (n=10), DUP-PST1-1175 (n=1), DUP-PST1-1140 (n=1) ir DUP-PST1-1163 (n=1). Kiaulių pernešama *C. Coli* pasižymi didele įvairove tiek tarp skirtingų bandų, tiek ir bandos viduje.

Raktažodžiai: *Campylobacter*, įvairovė, genotipavimas, prietaisas „RiboPrinter®“, kiaulės.

Introduction. Thermophilic campylobacters, represented by *Campylobacter jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis*, are zoonotic with many animals serving as reservoirs for human diseases including rabbits, rodents, wild birds, sheep, horses, cows, pigs, poultry, and domestic pets (Rosef et al. 1983, Kapperud and Rosef 1983, Brown et al. 2004, Johnsen et al. 2006a). The incidence of human cases of *Campylobacter* infection has increased and recently exceeded that of *Salmonella* in many European countries (<http://www.efsa.europa.eu/en.html>) and is the most frequently reported cause of bacterial gastrointestinal illness.

In industrialized countries, cases of human campylobacteriosis due to *C. jejuni* and *C. coli* are about 90% and 10% respectively. Despite the high number of human cases, knowledge of the pathogenesis and epidemiology of infections is still incomplete. Pigs are natural reservoirs of campylobacters, and isolation rates have been found to be between 77.6% and 100%, dominated by *C. coli* with 90.1 to 100% (Rosef 1981^{ab}, Rosef et al. 1983, Guevrement et al. 2004, Boes et al. 2005). *Campylobacter* are

apparently fragile organisms that are unable to grow in the presence of air or multiply outside the animal host and are highly susceptible to a number of environmental conditions (Park 2002). Murphy et al. (2006) summarize the mechanisms by which *Campylobacter* spp. adapt to stress conditions and thereby increase their ability to survive on food and in the environment.

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. Using genotyping to determine genetic relatedness is necessary to provide data for a better understanding of the epidemiological aspects. The purpose of this study was to identify the thermophilic campylobacters isolated from pigs to species level and to use the automated *PstI* ribotyping method (restriction enzyme polymorphism analysis of automated microbial characterization system) to provide evidence of genetic

relatedness. We used a highly standardized method coupled with a computer-based pattern analysis in order to compare the strains, which then provided the possibility to compare ribotype profiles and the strains in a dendrogram.

Material and methods

Bacterial isolates

Faecal swabs (Invasive sterile Eurotube® Collection Swab) from pigs (n=100) representing 10 herds were collected at slaughter and streaked onto modified bloodfree CCDA-Preston agar (Oxoid CM 739, Oxoid SR 155, and Oxoid SR 2342 E). The plates were incubated at 42°C under microaerobic conditions using Oxoid CampyGen in an anaerobic jar. The plates were read after 24 and 48 hours and typical colonies confirmed by phase contrast microscopy. The isolates (n=88) were kept frozen (-70°C) in Microbank™ (Pro-Lab Diagnostics, Canada) until further examination.

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 µg 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 500 µL of water, centrifuged, and 5 µL of the supernatant used as a template in the polymerase chain reaction (PCR). The PCR-mix consisted of the species-specific primers and multiplex master mix (Qiagen GmbH, Hilden, Germany), water, and the template in a total volume of 50 µL. The PCR was run (Bio-Rad, iCycler) with the following conditions: 95°C for 6 min, 30 repetitions of 95°C for 30 sec, 56°C for 30sec, 72°C for 30 sec, 72°C for 7 min, and finally ending at 4°C. 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.

Ribotyping

Ribotyping was performed on 81 selected *C. coli* isolates using the DuPont 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 80°C 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 *PstI* enzyme (20.000U/ML, New England Biolabs), 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

determined when the corresponding patterns matched one of the patterns of the DuPont Identification library of the RiboPrinter® with a similarity of > 0.85. The *PstI* ribo-type patterns were automatically assigned a DuPont identification number (e.g. DUP-PST1-1182) by the RiboPrinter®, which was confirmed by visual inspection. The profiles were transferred and analyzed with the GelComparII® software (Bio Numerics, Applied Maths inc.) 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 and discussion

Out of 100 faecal swab samples, 88 thermophilic *Campylobacter* (88%), representing all the herds investigated, were isolated (Table 1). Both biochemical and multiplex PCR distinguished the isolates to species level (Table 2). Out of 88 isolates, 86 (97.7%) were *C. coli*, one was *C. lari*, and one was *C. jejuni* as shown in Table 2. Both *C. jejuni* and *C. lari* were isolated from the same herd. (Table 1). Pigs are healthy carriers of *C. coli* and is also isolated from pigs environment and as surface contaminant of pig carcasses (Rosef, 1981^{ab}) The low isolation rate of thermophilic campylobacters others than *C. coli* is in accordance with earlier findings (Rosef et al. 1983, Guévremont et al 2004, Boes et al. 2005).

Table 1. Isolation of *Campylobacter* species from the herds

Herds	Number of samples	<i>Campylobacter</i> Isolated
A	9	8
B	1	1
C	1	1
D*	39	31
E	1	1
F	1	1
G	5	5
H	1	1
I	15	13
J	27	26

* One isolate of *C. jejuni*, and one *C. lari* was isolated from herd D.

Although thermophilic campylobacters do not 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). 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 and cause infections. At present, this is unknown. The infective dose, however, is very low, with only a few hundred bacteria necessary to cause human infection (Black et al. 1988). The high infection rate indicates that hygienic precautions should be taken when handling pigs.

Table 2. Distribution of *Campylobacter* species by using biotyping and multiplex PCR

Species	<i>Campylobacter</i> multiplex PCR	Biotyping		%	DuPont ID
		Hippurate	Nalidixic acid		
<i>C. jejuni</i>	1	1	0	1,1	
<i>C. coli</i>	86	0	0	97,7	19
<i>C. lari</i>	1	0	1	1,1	
Total	88	1	1	100	19

Earlier works on the serotyping of *C. spp.* isolated among domestic and wild animals (including pigs) have displayed diversity with 42 different serotypes among the typeable strains (65,7%) (Rosef et al.1985). Knowledge of the virulence and diversity of the strains isolated from pigs is lacking. We used the restriction enzyme *Pst*I for automatic ribotyping because of the Qualicon library identification (DUP-Pst ID) in the RiboPrinter® and because of the use by other researchers (de Boer 2000, Ge et al. 2006). It is then possible to compare the ribotype pattern with other RiboPrinter® users. A number of different ribotypes were found within *C. coli*. The ribotypes could be divided into two clades and five subclades (a-e) as showed in Figure 1. Five strains did not fall into clusters. Nineteen isolates could be given five different Dup-IDs from the library. DUP-PST1-1182 (n=5), DUP-PST1-1208 (n=10), and DUP-PST1-1175 (n=1) - representing 16 of the DUP-IDs classified - were in subcluster b originating from five herds. Subcluster e with 15 isolates represents one herd. One of these strains was given a DUP-ID (DUP- PST1-1140). One strain in subcluster d was assigned DUP-PST1-1163 (Figure 1).

It could be concluded that there is a high diversity of the *C. coli* isolates among and within the herds. There are, however, few common ribotypes between isolates from human cases and pigs (Rosef et al. not published). 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 the same ribogroup as clinical isolates. Due to the lack of detectable virulence factors and the high number of isolates from pigs, special precautions should be taken to avoid the risk of transmitting campylobacteriosis.

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