REPETITIVE ELEMENT SEQUENCE-BASED PCR TYPING FOR IMPROVED DISCRIMINATION OF *CAMPYLOBACTER JEJUNI*

Mindaugas Malakauskas[1,*], *Alvydas Malakauskas*[3], *Henrik Christensen*[2], John Elmerdahl *Olsen*[2], *Carl Henrik Brogren*[4]

[1]*Department of Food Safety and Quality, Faculty of Veterinary Medicine, Veterinary Academy, Lithuanian University of Health Sciences, Tilžės str. 18, LT-47181, Kaunas, Lithuania*
[2]*Department of Veterinary Pathobiology, Faculty of Life Sciences, University of Copenhagen, Groennegaardsvej 15, DK-1870 Frederiksberg C, Denmark*
[3]*Department of Veterinary Pathobiology, Faculty of Veterinary Medicine, Veterinary Academy, Lithuanian University of Health Sciences, Tilžės str. 18, LT-47181, Kaunas, Lithuania*
[4]*Department of Biomedical Sciences, Faculty of Health Sciences, University of Copenhagen, Blegdamsvej 3, DK-2200 Copenhagen N, Denmark*

[*]*Corresponding author: Mindaugas Malakauskas*
Lithuanian University if Health Sciences, Tilzes str. 18, 47181, Kaunas, Lithuania
Phone/fax: +370 37 362 695; e-mail: mindaugas.malakauskas@lsmuni.lt

Abstract. The aim of our studies was to evaluate rep-PCR typing using different primers as a fast genotyping method for discrimination of *C. jejuni*. Also a microfluidic electrophoretic DNA separation method for rapid DNA fingerprinting based on Lab-on-a-Chip technology was tested.

Based on the test results, the single polytrinucleotide $(GTG)_5$ was chosen as primer. Nine strains representing three epidemiological groups from the CAMPYNET network strains were assigned to three different groups by both $(GTG)_5$-PCR and PFGE. Typing of 72 epidemiologically independent strains using $(GTG)_5$-PCR and PFGE clustered the strains in 60 and 53 clusters, respectively, with an identical discriminatory power (D=0,99). When PCR-products were separated on the Agilent 2100 Bioanalyzer Lab-on-a-Chip device the strains were grouped with the same discriminatory power, though some strains were clustered differently compare to gel-based DNA amplicon separation. The main disadvantage of $(GTG)_5$-PCR typing was differences in band intensity that complicates comparison of $(GTG)_5$-PCR fingerprints when numerous strains are evaluated. The advantages of rep-PCR based subtyping were rapidity, simplicity, similar discriminatory power to PFGE and possible rapid DNA amplicon separation based on Lab-on-a-Chip technology with Agilent 2100 Bioanalyzer.

Keywords: *Campylobacter*, rep-PCR, genotyping.

Introduction. Molecular methods with high discriminatory power are essential tools for reliable typing of thermophilic *Campylobacter* species, especially when high-quality data are required to estimate the transmission routes during outbreaks. The most commonly used typing methods (pulse field gel electrophoresis (PFGE), ribotyping, flagellin gene typing, multilocus sequence typing (MLST), random amplified polymorphic DNA typing (RAPD); serotyping, phage typing, biotyping) differ in discrimination ability, typeability, automatisation level, reproducibility and some other characteristics (Steele et al., 1998; Nielsen et al., 2000; Ono et al., 2003). Currently, PFGE is considered the golden standard for outbreak investigations, even though it is rather slow and laborious (Cloak and Fratamico, 2002; Hume et al, 2002).

Fast PCR-based methods are often preferred in situation where a rapid response to typing results is needed. Versalovic *et al.* (1991) first described the use of repetitive element sequence-based PCR (rep-PCR) for direct fingerprinting of bacterial genomes. It is based on the fact that outwardly facing primers, complementary to interspersed repeated sequences, enable the amplification of different size DNA fragments, consisting of sequences lying between these elements. Very well described examples of these repetitive elements sequences are the repetitive extragenic palindromic (REP) sequences, the BOX sequences, the enterobacterial repetitive intergenic consensus (ERIC) sequences and the polytrinucleotide $(GTG)_5$ sequence (Versalovic et al., 1994). Rep-PCR typing method using different primer(s) has been successfully used to differentiate strains of *Streptococcus pneumoniae* (Harrington et al. 2007), *Escherichia coli* (Goldberg et al. 2006), *Bacillus thuringiensis* (Reyes-Ramirez and Ibarra, 2005), *Staphylococcus aureus* (Ross et al. 2005), salmonella isolates (Rasschaert et al. 2005; Weigel et al. 2004), *Vibrio parahaemolyticus* (Malupin et al. 2005), *Clostridium difficile* (Northey, et al. 2005) and others.

Rep-PCR typing has been applied for typing of campylobacters and was shown to be less discriminative than PFGE (Steinbrueckner et al. 2001), Fourier Transform Infrared Spectroscopy (Mouwen et al. 2005), and RFLP (Weijtens et al. 2000). This method has been applied for typing of a limited number of *Campylobacter* strains using commercially available kits from Bacterial Barcodes (Athens, GA, USA) (Hiett et al. 2003; Hiett et al. 2006). There are, however, no published studies, where rep-PCR typing has been compared to PFGE

typing of a representative number of *Campylobacter* strains.

The aim of our studies was to evaluate rep-PCR typing using different primers, as a fast genotyping method in comparison to PFGE for discrimination of *C. jejuni* isolates from different sources. Also we have tested a microfluidic capillary electrophoresis for rapid DNA fingerprinting on a chip-based technology with Agilent 2100 Bioanalyzer.

Methods and Materials

Strains used in the study. Strains of campylobacters (2683; 2686; 2759; 29406; 4129; 4133; 4135) from the collection of Department of Veterinary Pathobiology, Faculty of Life Science, University of Copenhagen, Denmark and from the CAMPYNET network strain collection (strain numbers are original used by Harrington et al, 2003) were used for selection of primers. Seventy two epidemiologically independent strains of *C. jejuni* from the strain collection described by Nielsen *et al.* (2000) were used to calculate discriminatory power of rep-PCR and PFGE methods.

Bacterial culture and DNA extraction. Bacterial cultures were grown at 37 °C for 48 hours on Blood Agar Base No. 2 (Oxoid, CM0271, Hampshire, England) with 7% blood (dried for 6-7 hours) under microaerophilic conditions. Single colonies from each plate were taken to inoculate new plates and these were grown as described, except the plates were not dried. Bacteria were scraped of the plate for DNA extraction. DNA used for comparison of rep-PCR and PFGE originated from the same plate.

Bacteria (half of one 10 µl loop) were suspended in 500 µl 5% Chelex solution (BioRad, Hercules, USA) and mixed carefully, incubated at 56 °C for 30 min, shaken and boiled for 10 min. The tubes were shaken again for 5–10 s and centrifuged 2.5 min at 10000 rpm. The supernatants were carefully transferred into new tubes and finally cleared by repeated centrifugation. Supernatants were stored at −20 °C before use. DNA template was quantitated using a spectrophotometer (GeneQuant RNA/DNA Calculator; Pharmacia Biotech (Biochrom) Ltd., Science Park, Cambridge CB4 4FJ, England)).

Bioinformatic investigations and selection of primers for rep-PCR. Seven different primers were tested in the study (Table 1). Three primers were described previously by Versalovic et al. (1991). Four repeated sequence primers were designed on the basis of a bioinformatic investigation of the published genomic sequences of *C. jejuni* and *C. coli* isolates with accession numbers NC_003912 and NC_002163, respectively (Wheeler et al. 2007). They were searched for repeated sequences by use of the PERL program n-mer_PM.pl published by Bakkali et al. (2004). Two forward primers and two reverse primers were then selected based on the criteria of 1) conservation for the 3' ends with respect to the two genomes, 2) the 3' end should be a purine, 3) the span between forward and reverse primers should be possible to resolve by traditional agarose-gel electrophoresis i.e. between 100 – 4000 bp, 4) the match in T_m between forward and reverse primers should be within 5 ˚C and 5) the primer length should be minimum 14 bp.

Table 1. **Primers used in the study**

Primers	Primer sequence
REP1R-Dt	5'-IIINCGNCGNCATCNGGC-3' (Versalovic et al., 1991)
REP2-Dt	5'-NCGNCTTATCNGGCCTAC-3' (Versalovic et al., 1994)
GTG5	5'-GTGGTGGTGGTGGTG-3' (Versalovic et al., 1994)
REPICJF	5'-TYTTYTAAAATTTC-3' (this study)
REPIICJF	5'-AAAAAATTTAAAAGC-3' (this study)
REPICJR	5'-TCTTTTAAAATTTC-3' (this study)
REPIICJR	5'-TTTAAAATTTCATC-3' (this study)

PCR conditions. A master mixture consisting of all reagents except genomic DNA and *Taq* DNA polymerase was prepared and aliquoted in tubes each holding 45 µl. PCR was initiated by adding 2500 ng of genomic DNA to each tube and 2 U of *Taq* DNA polymerase (*Thermus aquaticus*) (GE Healthcare, UK). Each 45µl of the master mixture contained 0.76 µl of 10x PCR Buffer; 1.9mM of $MgCl_2$, a mixture of deoxynucleoside triphosphates at 11.8 mM of each and primers at 20 µM.

The PCR conditions varied with respect to annealing temperature and cycling conditions. The final protocol included an initial denaturation step (94°C, 2 min), 31 cycles of amplification (94°C, 30 s, 40°C, 60s min, 72°C, 110s) followed by one final extension step at 72°C for 16 min. Fifteen microliters of amplification products was electrophoresed in 0.5xTBE at 100 V in a 1.3% agarose gel (SeaKem LE Agarose, Cambrex Bio Science, Rockland Inc., Rockland ME USA). MassRuler™ DNA Ladder Mix (Fermentas) was loaded in appropriate wells. The gels were stained with 10 mg of ethidium bromide per ml for 15 min, destained in water for 15min, and then photographed with a UV light source in Gel Doc 2000 system (BioRad, Hercules, USA).

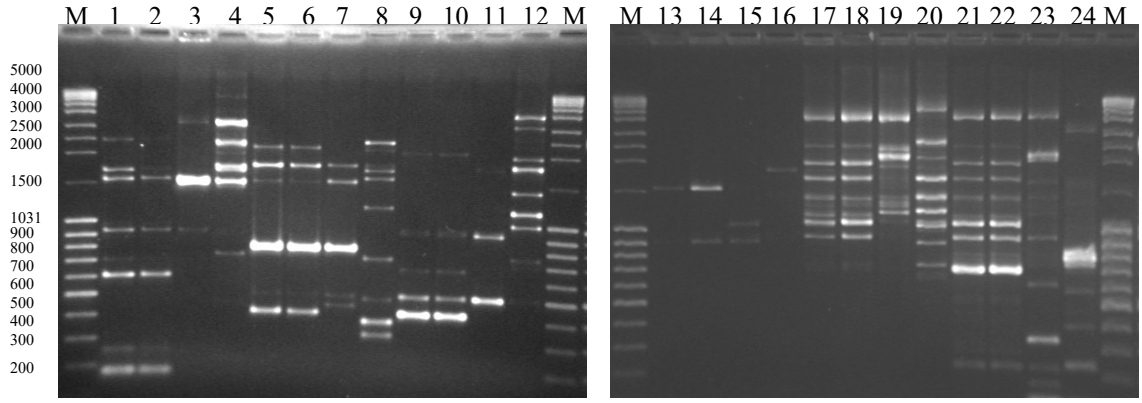
PFGE conditions. Subtyping of isolates was done using macrorestriction enzyme *Sma*I (Amersham Pharmacia Biotech, E1085W, China) as described by Ribot *et al.* (2001) with minor changes as follows. Adjustment of the concentration of cell suspension was made using standard McFarland tube No. 2. Lambda Ladder PFG Marker (New England BioLabs Inc., N0340S, Beverly, MA) was used as the molecular size marker. In addition, similar PFGE patterns with slight difference in band position were compared by running electrophoresis on the same gel.

Analysis of $(GTG)_5$-PCR and PFGE patterns. After electrophoresis, the $(GTG)_5$-PCR and PFGE patterns were analysed using GelCompar II software (Applied Maths BVBA, Sint-Martens-Latem, Belgium). The saved gel images were converted to tif-files and normalized by aligning the peaks of the size molecular marker, which was loaded every six sample lines in each gel. Matching and dendogram UPGMA (unweighted pair group method with averages) analysis of patterns was performed using the Dice coefficient with a 1% band position tolerance window. In addition all patterns were compared manually.

Separation of GTG_5-PCR amplicons by microfluidic capillary electrophoresis on chip-based technology with Agilent 2100 Bioanalyzer. In order to test the possibility for semi-automatisation and rapid DNA fingerprinting, amplicons from $(GTG)_5$-PCR of all tested strains in these study were analysed by separation of amplified DNA on the DNA 7500 Assay Kit with Agilent 2100 Bioanalyzer (Agilent Technology, Palo Alto, California). The separation performed according Reagent Kit Guide of DNA 7000 Assay. Shortly, Gel-Dye Mix, Markers and Ladder were loaded in appropriate wells of the DNA chip as recommended in the guide. 1µl of each sample were loaded in each of the sample wells and the chips were placed in the adapter and vortexed for 1 min. at 2400rpm. Finally, the chip was run in the Agilent 2100 Bioanalyzer within 5 min. The gel like images were stored as tiff-files and patterns were analysed using GelCompare II software (Applied Maths BVBA, Sint-Martens-Latem, Belgium) as described above.

Calucalation of discriminatory power. Similarity values of 95% were used to distinguish between $(GTG)_5$-PCR profiles and types were arbitrarily defined on that basis. Any differences among PFGE profiles were considered significant, and types were arbitrarily defined on that basis. Discriminatory power (D) was calculated as described by Gaston and Hunter (1989).

Results. Selection of primers. We tested seven different primers (Table 1) in six combinations under different PCR conditions. The primers were initially tested against 3 strains of *C. jejuni* and one strain of *C. coli* for the ability to produce a suitable number of bands of a conventional size. Figure 1 shows results for the PCR conditions that gave the best banding pattern for each PCR method.



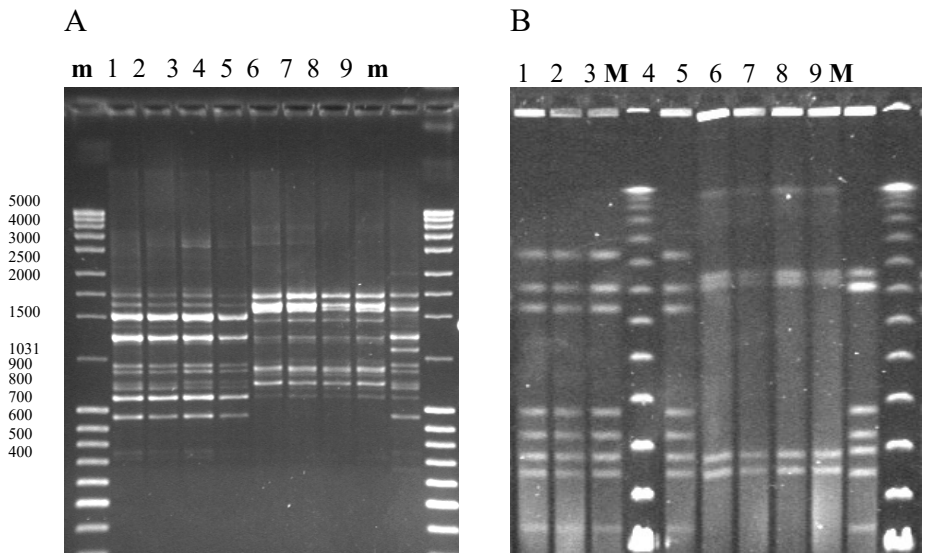
Campylobacter jejuni No 1. – lines 1,5,9,13,17 and 21; *C. jejuni* No 2. – lines 2,6,10,14,18 and 22; *C. jejuni* No 3 – lines 3,7,11,15,19, and 23; *C. coli* No 4 – lines 4,8,12,16,20 and 24. Primers sets: lines 1-4 – REPICJF/REPIICJR; lines 5-8 – REPIICJF/REPIICJR; lines 9-12 – REPIICJF/REPICJR; lines 13-16 – REP1R-Dt/REP2-Dt; lines 17-20 – $(GTG)_5$; and lines 21-24 – REP1R-Dt/REP2-Dt/$(GTG)_5$. M - MassRuler™ DNA Ladder Mix (Fermentas).

Fig. 1. **Testing of different primer combinations for rep-PCR typing of *Campylobacter***

The different PCR-methods produced from one and nine bands in the size range of 200 to 4000 bps. The best patterns were obtained when the primer $(GTG)_5$ was used as a single primer. It produced 9 fragments for *C. jejuni* and *C. coli* in the range from about 700bp to about 4000 bps, except one strain when only 6 fragments were produced. The use of the primers REP1R-Dt/REP2-Dt/$(GTG)_5$) in a mixture also produced a suitable pattern for typing, consisting of 7 fragments, but with quite week fragments from one strain of *C. jejuni* and the *C. coli* strains. The results were reproducible when the same PCR and electrophoresis conditions were applied.

Based on the results, the single polytrinucleotide $(GTG)_5$ was chosen as primer for further tests using strains *C. jejuni NCTC 11168*, C. jejuni, *C. coli* NCTC 11353, *C. coli* and *C. lari* NCTC 11352 and *C. lari* CCUG 29406 and compared to PFGE profiles of the same strains. With both methods all six campylobacters strains generated different profiles after visual comparison (data not shown). The obtained $(GTG)_5$ -PCR profiles were most similar within the individual species. Also PFGE fingerprints of the same campylobacter species were most similar to each other in bands number and position.

$(GTG)_5$-PCR and PFGE typing of outbreak strains. Typing of nine strains representing three epidemiological groups from the CAMPYNET network strains collection showed the discriminatory potentiality of $(GTG)_5$-PCR typing compared to PFGE. Both methods assigned outbreak strains to three different groups (Fig. 2). Profiles obtained by both $(GTG)_5$-PCR and PFGE typing were identical within outbreak strains and different among epidemiologically not related sets.



A - $(GTG)_5$-PCR typing; M - MassRuler™ DNA Ladder Mix (Fermentas); B – PFGE typing; M - Lambda Ladder PFG Marker; Line 1 – CNET 001, 2 – CNET 002, 3 – CNET 003, 4 – CNET 004, 5 – CNET 005, 6 – CNET 006, 7 – CNET 007, 8 – CNET 008, 9 – CNET 009) Strains 001-004 represent outbreak 1; 005-008 – outbreak 2 and 009 – outbreak 3. Strain numbers are original used by Harrington et al, 2003.

Fig. 2. **$(GTG)_5$-PCR and PFGE typing of outbreak strains of *C. jejuni***

Discriminatory power of $(GTG)_5$-PCR and PFGE subtyping methods. Typing of 72 epidemiologically independent strain using $(GTG)_5$-PCR and PFGE clustered strains to in 60 and 53 clusters respectively (Fig 3 and 4). There were six clusters of two strains and two clusters of four strains with identical $(GTG)_5$-PCR profiles. The other 52 strains generated different profiles and were clustered separately. PFGE typing generated two clusters of 4 strains, four clusters of 3 strains, and five clusters of 2 strains with identicasl profiles. The other 42 PFGE profiles were represented by one strain only. The discriminatory power (D) was identical for $(GTG)_5$-PCR and PFGE (D=0,99). $(GTG)_5$-PCR and PFGE typing agreed in most cases on grouping of strains but for 16,7% of the strains tested, the methods clustered them differently.

Separation of GTG_5-PCR amplicons by microfluidic capillary electrophoresis The strains were grouped in a similar manner and with the same discrimination when the PCR-products were separated on the Agilent 2100 Bioanalyzer Lab-on-a-Chip device (Fig. 5). There were one cluster of six strains with identical profiles, one cluster of three strains and thirteen clusters of two identical strains. Table 2 summarises evaluation of the methods with the respect to discriminatory power, number of types detected and number of unique type.

Table 2. **Typing of 72 *C. jejuni* isolates by $(GTG)_5$-PCR and PFGE**

Typing methods	No. of types	No. of unique types[a]	D[b]
$(GTG)_5$-PCR			
gel based	60	52	0,99
chip based	51	37	0.99
PFGE	53	42	0,99
$(GTG)_5$-PCR(gel)+PFGE	67	62	0,99
$(GTG)_5$-PCR(chip)+PFGE	68	64	0,99
[a]-Number of types represented by only one isolate; [b]- Discriminatory power (D) was calculated as described by Gaston and Hunter (1989).			

Discussion. There is a need for better and more rapid typing methods for large scale epidemiological investigations of *Campylobacter* strains. Promising results with rep-PCR on other bacterial species such as *Salmonella* (Rasschaert et al; 2005), lactic acid bacteria (Gevers et al. 2003), *Saccharomyces cerevisiae* (Caruso et al. 2002) and *Enterococcus* spp (Svec et al. 2005) lead us to investigate this method for typing of campylobacters. Also we explored the possibility for scoring of typing results in a cheap semi-automated rapid technology using a microfluidic electrophoretic version based on the now commercial available Lab-on-Chip technology.

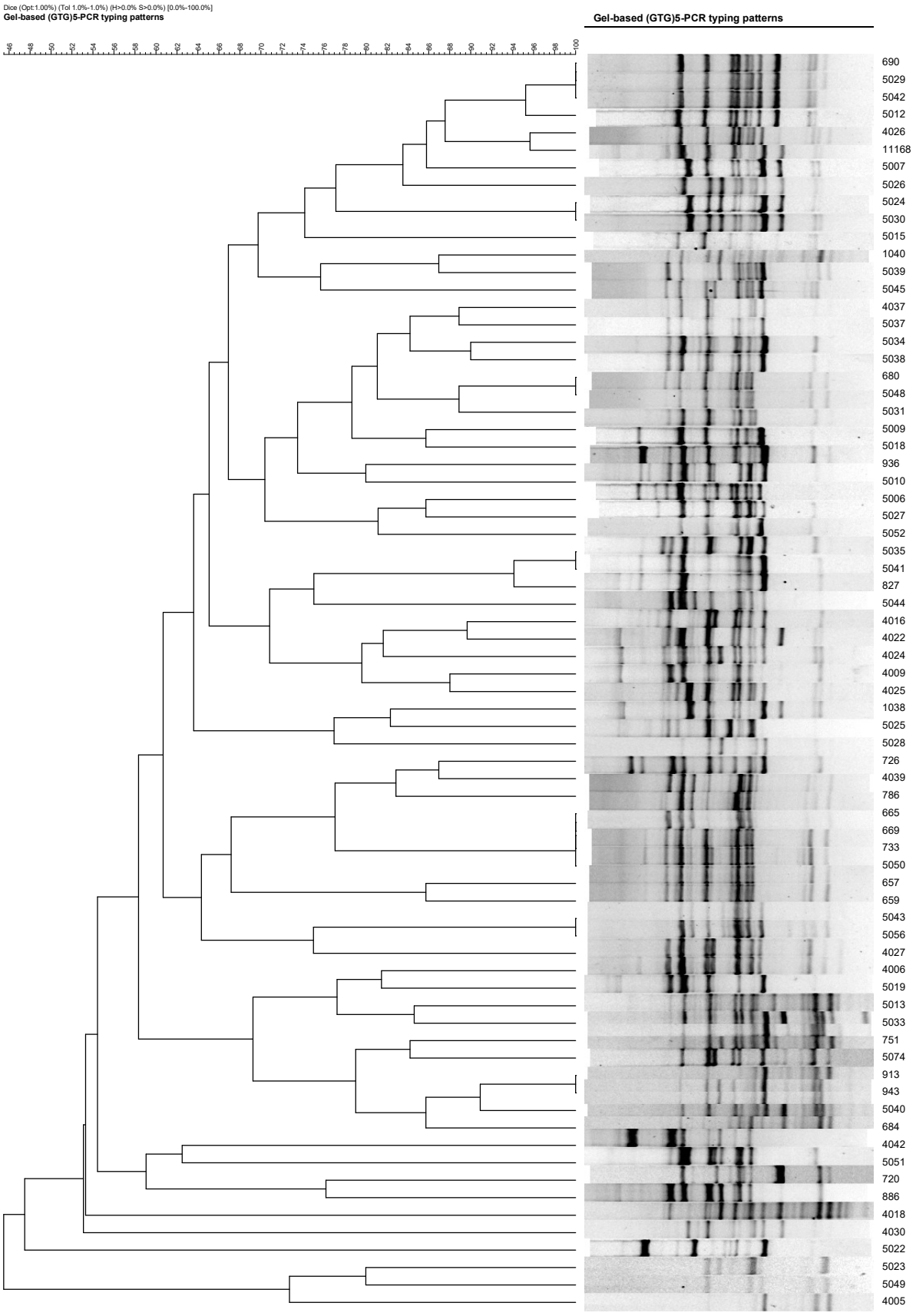


Fig. 3. **Dendogram showing clustering of *C. jejuni* strains by Gel-based (GTG)$_5$-PCR typing (number 11168 represents *Campylobacter jejuni* NCTC 1116)**

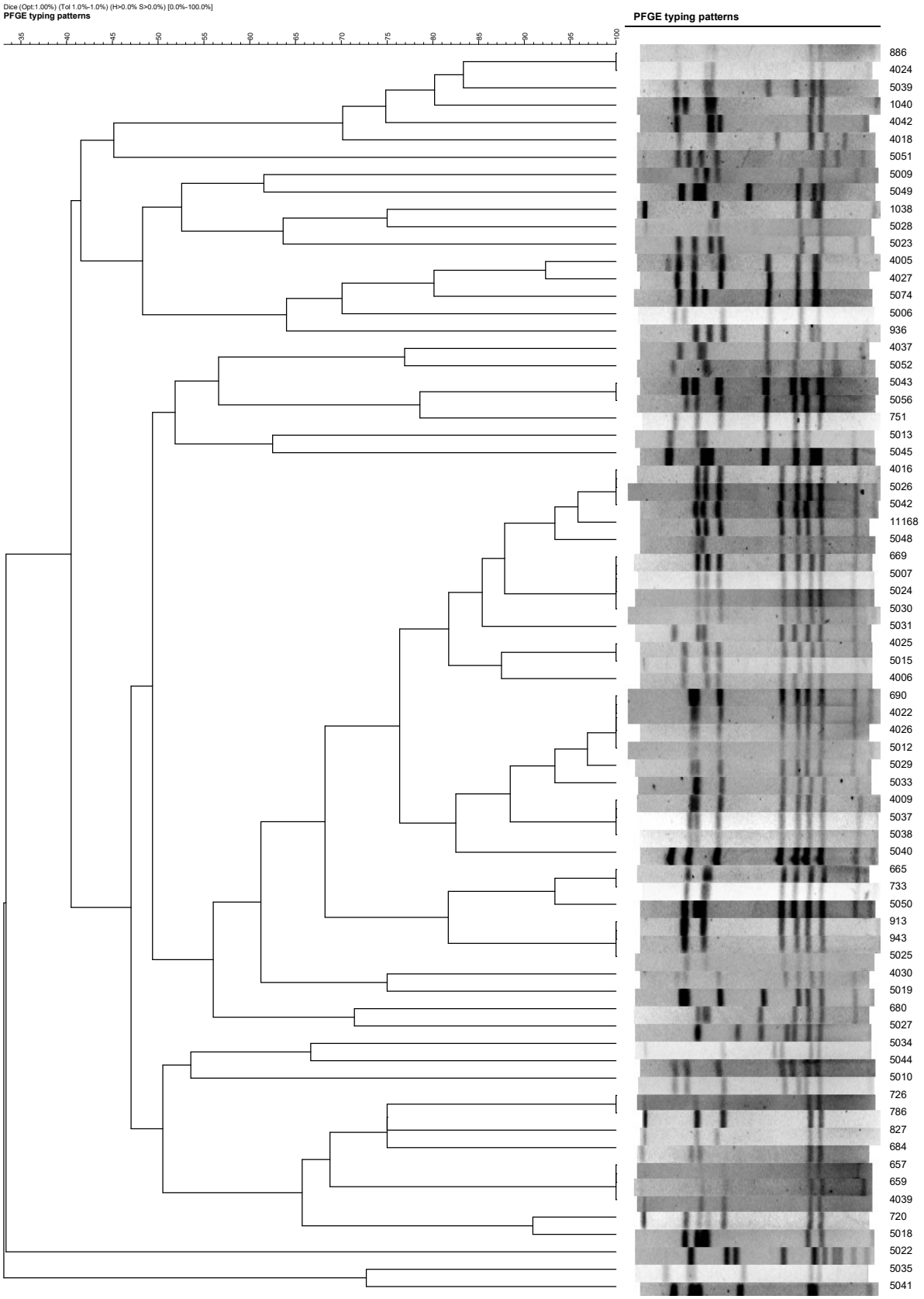


Fig. 4. **Dendogram showing clustering of *C. jejuni* strains by PFGE typing (number 11168 represents *Campylobacter jejuni* NCTC 1116)**

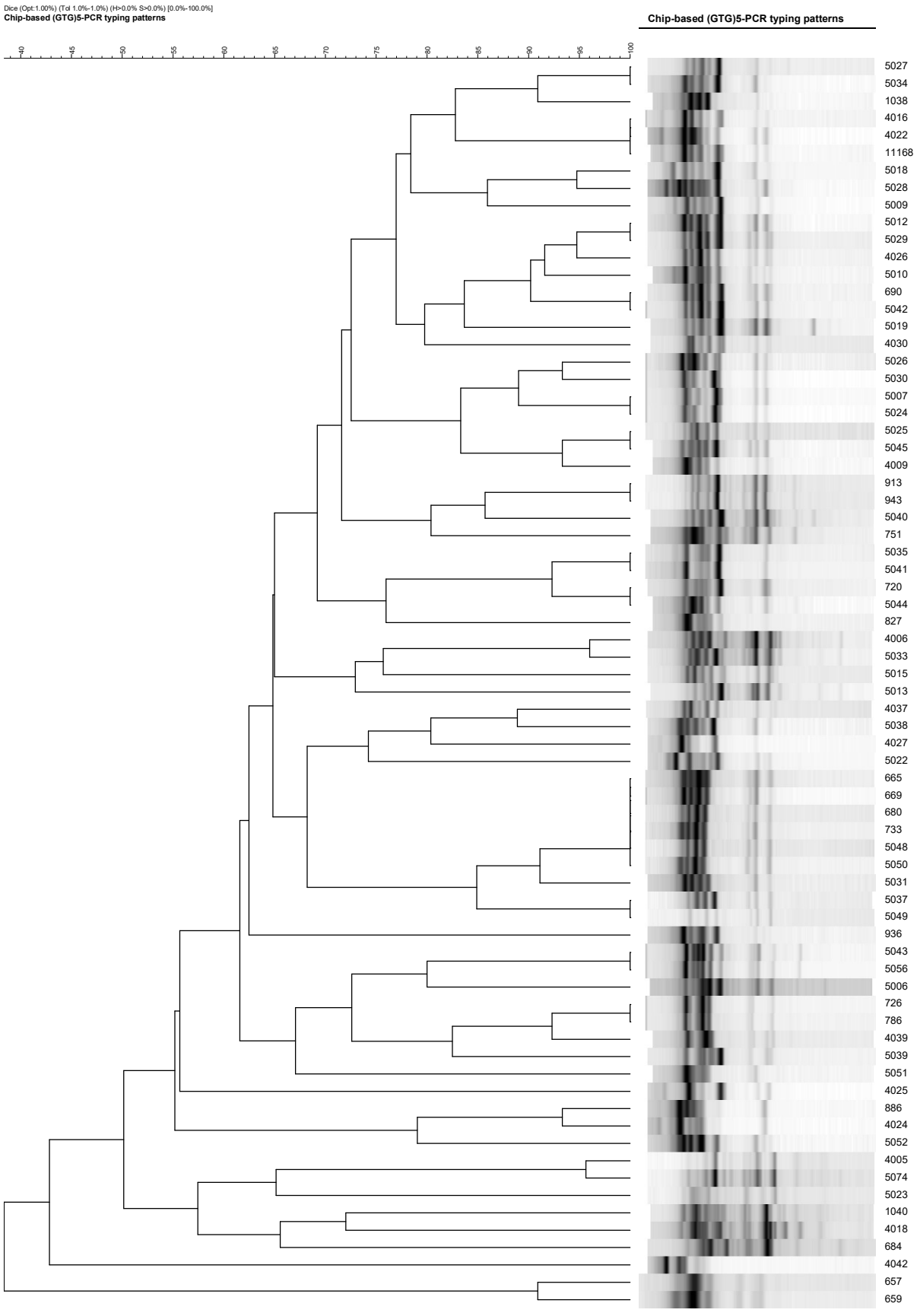


Fig. 5. **Dendogram showing clustering of *C. jejuni* strains by Chip-based $(GTG)_5$-PCR typing (number 11168 represents *Campylobacter jejuni* NCTC 1116)**

Rep-PCR has previously been applied for typing purpose of a limited number of strains of *Campylobacter* using ERIC primers. The results of these studies showed that ERIC-PCR typing is less discriminatory than fourier transform infrared spectroscopy (Mouwen et al., 2005), PFGE (Steinbrueckner et al., 2001) and RFLP (Weitjtens et al. 2000). Therefore the present study included a search for more optimal primers among already published rep-PCR primers and repetitive sequences in the genomes of *C. jejuni* and *C. coli*. Among several already published rep-PCR primers and the sequences designed in the present study (REPICJF, REPIICJF, REPICJR, REPIICJR), the most discriminatory typing results were obtained using the single $(GTG)_5$ rep-PCR primer. Primer testing included modification of the PCR protocol with respect to annealing temperature and time-cycles features (data not shown) and repeatedly the $(GTG)_5$-PCR was the most robust and reproducible method.

The primers designed in the present study were based on the genomic sequences published of *C. jejuni* and *C. coli* with a perfect match to the target sequence as a main criterion. Although three out of the four primer combinations produced clear and reproducible fragments with the four reference strains tested, less fragments were observed compared to the $(GTG)_5$ primer. This primer does not have a perfect match to any of the two published genomic sequences; however, its high efficiency of amplification is probably related to high tolerance for binding with purine rich regions in the genomes as well as pyrimidines. G in the 3' end of the primer is expected to allow amplification by binding to T as well as C in the template and even give weak amplification with G in template (Kwok *et al.* 1990).

Previous studies (Hiett et. al. 2003; Hiett et al. 2006) have shown that rep-PCR can discriminate between strains of *C. jejuni* and *C. coli* strains using a commercial kits (Bacterial Barcodes, Athens, GA, USA). The sequence of primer(s) used in this system is, however not publically available. The results of the latest study conclude that rep-PCR using commercial typing kits is less discriminatory compare to DNA sequence analysis of *fla*A SVR (Hiett et al. 2006).

Rep-PCR based typing with the $(GTG)_5$ primer was rapid, easy to perform and showed high discriminatory. This is in line with several studies with other bacteria which have demonstrated that rep-PCR has considerably better discriminatory power than restriction analysis of the 16S rRNA gene (Appuhamy et al. 1997) the 16S-23S spacer region (Vila et al. 1996) multi locus enzyme electrophoresis (Woods et al. 1992), biochemical characterizations (Clarridge et al. 1995), and ribotyping (Snelling et al. 1996). Some studies have recommend rep-PCR (using BOX, ERIC, and REP) primers as the preferred method for tracing of transmission routes for *Salmonella* after comparison to PFGE typing (Weigel et al. 2004). Also resent studies showed that $(GTG)_5$ oligonucleotide was a useful primer type *Campylobacter concisus* allowing for discrimination between epidemiologically related and unrelated strains (Matsheka et al. 2006).

It was expected to see identical PFGE genotyping data being further differentiated by the rep-PCR analysis, but we also saw the opposite; identical rep-PCR pattern were demonstrated from strain which easily could be differentiate by PFGE. This means that application of both methods in parallel will increase discriminatory power. As example the reference strain *C. jejuni* NCTC 11168 was clustered differently by the two methods.

Promising results were obtained with Agilent 2100 Bioanalyzer for DNA separation instead of running gel electrophoresis. These results confirm the ability of this system with respect to sizing resolution and accuracy of DNA separation (Nachmkin et al., 2001; Panaro et al., 2000). Even though the results were promising, problems were observed. The commercially available DNA 7500 Kit did not separate all closely sized DNA fragment. In many cases DNA fingerprints had up to 4 bands less compared to fingerprints generated after conventional gel based separation. The same disadvantage related to unresolved DNA fragments was also noticed by Nachamkin *et al.* (2001). Our observations show that it would be possible to improve the gel-matrix for this particular rep-PCR application, since the lowest range and the highest show no band at all (data not shown), and could be excluded from the recording range by using an alternative set of high and low markers, which were not available commercially at the studies time. By developing such DNA separation kit within range from about 500bp to about 4000bp the band patterns would not be changed but the visual evaluation improved by expanding the distance between band that might also improve further resolution. We propose that microfluidic separation of DNA on a chip has the potential to replace conventional DNA separation if an appropriate DNA separation kit is used.

The discriminatory power was equally high in separation by agarose gel electrophoresis. Similar high D was established also by Nielsen et al. (2000) then the same strain collection were typed by PFGE (D=0.974), RAPD (D=0.984) and calculated combined discriminatory power (D=0.998) respectively.

The main disadvantage of $(GTG)_5$-PCR fingerprints was differences in band intensity, reflecting different efficiency of DNA amplification. This complicates comparison of $(GTG)_5$-PCR fingerprints when numerous strains are evaluated. To achieve the best quality DNA fingerprints we studied the impact of genomic DNA template quantity on the reproducibility and the quality of GTG-PCR fingerprint patterns and found that within certain range of quantity (50-5000ng) the reproducibility was high with minor changes in the intensity of both big and small DNA fragments (data not shown). Such stability was also observed by other authors (Healy et al., 2005). The few cases where $(GTG)_5$-PCR fingerprints were not reproducible included small (less then 600bp) and big (more than 4000bp) DNA bands. They were always amplified but the intensity varied. We therefore chose to evaluate all patterns according to the number of DNA fragments and their position. Some authors propose to evaluate the intensity differences of individual bands in

the pattern (Hiet et al., 2006), however in the current study that would have resulted in lower reproducibility..

In summary the advantages of $(GTG)_5$-PCR based subtyping was rapidity, simplicity, similar D to PFGE and possible automatization using Lab-on-a-Chip technology. Some PFGE protocols enable complete subtyping within 30 h (Gautom, 1997; Lukinmaa et al, 2004). In comparison, rep-PCR allows fingerprinting in about 9 -10 hours running standard gel electrophoresis and in 6-7 h using chip-based microfluidic electrophoretic separation.

Acknowledgements

This study was supported by the Danish Ministry of Science, Technology and Innovation through a Danish Governmental Scholarship to the first author to cover living expenses during stay at the Department of Veterinary Pathobiology, University of Copenhagen.

The authors would like to thank the technical stuff of the Department of Veterinary Pathobiology, University of Copenhagen for excellent assistance during this study. Also we would like specially to thank Eva M. Nielsen (State Serum Institute, Copenhagen, Denmark) and Stephen L. W. On (Christchurch Science Centre, Christchurch, New Zealand) for sharing their strains; Anders Miki Bojesen from Department of Veterinary Pathobiology, University of Copenhagen, for help with numerical analysis.

References

1. Appuhamy, S., R. Parton, J. G. Coote, and H. A. Gibbs. 1997. Genomic fingerprinting of *Haemophilus somnus* by a combination of PCR methods. J. Clin. Microbiol. 35:288-91.

2. Bakkali, M., T. Y. Chen, H. C. Lee, and R. J. Redfield. 2004. Evolutionary stability of DNA uptake signal sequences in the *Pasteurellaceae*. Proc. Natl. Acad. Sci. U.S.A. 101: 4513-4518.

3. Caruso, M., A. Capece, G. Salzano, and P. Romano. 2002. Typing of *Saccharomyces cerevisiae* and *Kloeckera apiculata* strains from Aglianico wine. Lett. Appl. Microbiol. 34:323-328.

4. Clarridge, J. E., T. J. Raich, D. Pirwani, B. Simon, L. Tsai, M. C. Rodriguez-Barradas, R. Regnery, A. Zollo, D. C. Jones, and C. Rambo. 1995. Strategy to detect and identify *Bartonella* species in routine clinical laboratory yields *Bartonella henselae* from human immunodeficiency virus-positive patient and unique *Bartonella* strain from his cat. J. Clin. Microbiol. 33:2107-2113.

5. Cloak, O. M., and P. M. Fratamico. 2002. A multiplex polymerase chain reaction for the differentiation of *Campylobacter jejuni* and *Campylobacter coli* from a swine processing facility and characterization of isolates by pulsed-field gel electrophoresis and antibiotic resistance profiles. J. Food Prot. 65:266-273.

6. Gaston, M. A., and P. R. Hunter. 1989. Efficient selection of tests for bacteriological typing schemes. J. Clin. Pathol. 42:763-766.

7. Gautom, R. K. 1997. Rapid pulsed-field gel electrophoresis protocol for typing of *Escherichia coli* O157:H7 and other gram-negative organisms in 1 day. J. Clin. Microbiol. 35:2977-2980.

8. Gevers, D., L. Masco, L. Baert, G. Huys, J. Debevere, and J. Swings. 2003. Prevalence and diversity of tetracycline resistant lactic acid bacteria and their tet genes along the process line of fermented dry sausages. Syst. Appl. Microbiol. 26:277-283.

9. Goldberg T. L., T. R. Gillespie, and R. S. Singer. 2006. Optimization of analytical parameters for inferring relationships among *Escherichia coli* isolates from repetitive-element PCR by maximizing correspondence with multilocus sequence typing data. Appl. Environ. Microbiol. 72:6049-6052.

10. Harrington, C. S., L. Moran, A. M. Ridley, D. G. Newell, and R. H. Madden. 2003. Inter-laboratory evaluation of three flagellin PCR/RFLP methods for typing *Campylobacter jejuni* and *C. coli*: the CAMPYNET experience. J. Appl. Microbiol. 95:1321-1333.

11. Harrington S. M., F. Stock, A. L. Kominski, J. D. Campbell, J. C. Hormazabal, S. Livio, L. Rao, K. L. Kotloff, S. O. Sow and P. R. Murray. 2007. Genotypic analysis of invasive *Streptococcus pneumoniae* from Mali, Africa, by semiautomated repetitive-element PCR and pulsed-field gel electrophoresis. J. Clin. Microbiol. 45:707-714.

12. Hiett, K. L., G. R. Siragusa, N. A. Cox, R. J. Buhr, M. T. Musgrove, N. J. Stern, and J. L. Wilson. 2003. Genotype analyses of *Campylobacter* isolated from the gastrointestinal tracts and the reproductive tracts of broiler breeder roosters. Avian Dis. 47:406-414.

13. Hiett, K. L., B. S. Seal, and G. R. Siragusa. 2006. *Campylobacter* spp. subtype analysis using gel-based repetitive extragenic palindromic-PCR discriminates in parallel fashion to flaA short variable region DNA sequence analysis. J. Appl. Microbiol. 101:1249–1258.

14. Hume, M. E., R. E. Droleskey, C. L. Sheffield, and R. B. Harvey. 2002. *Campylobacter coli* pulsed field gel electrophoresis genotypic diversity among sows and piglets in a farrowing barn. Curr. Microbiol. 45: 128-132.

15. Kwok, S., D. E. Kellogg, N. McKinney, D. Spasic, L. Goda, C. Levenson, and J. J. Sninsky. 1990. Effects of primer-template mismatches on the polymerase chain reaction: human immunodeficiency virus type 1 model studies. Nucleic Acids Res. 18:999-1005.

16. Lukinmaa, S., U. M. Nakari, M. Eklund, and A. Siitonen. 2004. Application of molecular genetic methods in diagnostics and epidemiology of food-borne bacterial pathogens. APMIS. 112:908-929.

17. Maluping R. P., C. Ravelo, C. R. Lavilla-Pitogo, K. Krovacek, and J. L. Romalde. 2005. Molecular typing of *Vibrio parahaemolyticus* strains isolated from the Philippines by PCR-based methods. J. Appl. Microbiol. 99:383-391.

18. Matsheka, M. I., A. J. Lastovica, H. Zappe, and B. G. Elisha. 2006. The use of $(GTG)_5$ oligonucleotide as an RAPD primer to type *Campylobacter concisus*. Lett. Appl. Microbiol. 42:600-605.

19. Mouwen, D. J., M. J. Weijtens, R. Capita, C. Alonso-Calleja, and M. Prieto. 2005. Discrimination of enterobacterial repetitive intergenic consensus PCR types of *Campylobacter coli* and *Campylobacter jejuni* by Fourier transform infrared spectroscopy. Appl. Environ. Microbiol. 71:4318-4324.

20. Nachamkin, I., N. J. Panaro, M. Li, H. Ung, P. K. Yuen, L. J. Kricka, and P. Wilding. 2001. Agilent 2100 bioanalyzer for restriction fragment length polymorphism analysis of the *Campylobacter jejuni* flagellin gene. J. Clin. Microbiol. 39:754-757.

21. Nielsen, E. M., J. Engberg, V. Fussing, L. Petersen, C-H. Brogren, and S. L. On. 2000. Evaluation of phenotypic and genotypic methods for subtyping *Campylobacter jejuni* isolates from humans, poultry, and cattle. J. Clin. Microbiol. 38:3800-3810.

22. Ono, K., T. Kurazono, H. Niwa, and K. Itoh. 2003. Comparison of three methods for epidemiological typing of *Campylobacter jejuni* and *C. coli*. Curr. Microbiol. 47:364-371.

23. Panaro, N. J., P. K. Yuen, T. Sakazume, P. Fortina, L. J. Kricka, and P. Wilding. 2000. Evaluation of DNA Fragment Sizing and Quantification by the Agilent 2100 Bioanalyzer. Clinical Chemistry. 46:1851-1853.

24. Rasschaert, G., K. Houf, H. Imberechts, K. Grijspeerdt, L. De Zutter, and M. Heyndrickx. 2005. Comparison of five repetitive-sequence-based PCR typing methods for molecular discrimination of *Salmonella enterica* isolates. J. Clin. Microbiol. 43:3615-3623.

25. Reyes-Ramirez A. and J. E. Ibarra. 2005. Fingerprinting of Bacillus thuringiensis type strains and isolates by using Bacillus cereus group-specific repetitive extragenic palindromic sequence-based PCR analysis. Appl. Environ. Microbiol. 71:1346-1355.

26. Ribot, E. R., C. Fitzgerald, K. Kubota, B. Swaminathan, and T. J. Barrett. 2001. Rapid pulsed-field gel electrophoresis protocol for subtyping of *Campylobacter jejuni*. J. Clin. Microbiol. 39:1889-1894.

27. Ross T. L., W. G. Merz, M. Farkosh and K. C. Carroll. 2005. Comparison of an automated repetitive sequence-based PCR microbial typing system to pulsed-field gel electrophoresis for analysis of outbreaks of methicillin-resistant *Staphylococcus aureus*. J. Clin. Microbiol. 43:5642-5647

28. Snelling, A. M., P. Gerner-Smidt, P. M. Hawkey, J. Heritage, P. Parnell, C. Porter, A. R. Bodenham, and T. Inglis. 1996. Validation of use of whole-cell repetitive extragenic palindromic sequence-based PCR (REP-PCR) for typing strains belonging to the *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex and application of the method to the investigation of a hospital outbreak. J. Clin. Microbiol. 34:1193-1202.

29. Steele, M., B. McNab, L. Fruhner, S. DeGrandis, D. Woodward, and J. A. Odumeru. 1998. Epidemiological typing of Campylobacter isolates from meat processing plants by pulsed-field gel electrophoresis, fatty acid profile typing, serotyping, and biotyping. Appl. Environ. Microbiol. 64:2346-2349.

30. Steinbrueckner, B., F. Ruberg, and M. Kist. 2001. Bacterial genetic fingerprint: a reliable factor in the study of the epidemiology of human campylobacter enteritis? J. Clin. Microbiol. 39:4155-4159.

31. Svec, P., M. Vancanneyt, M. Seman, C. Snauwaert, K. Lefebvre, I. Sedlacek, and J. Swings. 2005. Evaluation of (GTG)5-PCR for identification of *Enterococcus spp*. FEMS Microbiol. Lett. 247:59-63.

32. Versalovic, J., T. Koeuth, and J. Lupski. 1991. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res. 19:6823-6831.

33. Versalovic, J., M. Schneider, F. de Bruijn, and J. Lupski. 1994. Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. Meth. Mol. Cell Bio. 5:25-40.

34. Vila, J., M. A. Marcos, and M. T. Jimenez de Anta. 1996. A comparative study of different PCR-based DNA fingerprinting techniques for typing of the *Acinetobacter calcoaceticus-A. baumannii* complex. J. Med. Microbiol. 44:482-489.

35. Woods Jr., C. R., J. Versalovic, T. Koeuth, and J. R. Lupski. 1992. Analysis of relationships among isolates of *Citrobacter diversus* by using DNA fingerprints generated by repetitive sequence-based primers in the polymerase chain reaction. J. Clin. Microbiol. 30:2921-2929.

36. Weigel, R. M., B. Qiao, B. Teferedegne, D. K. Suh, D. A. Barber, R. E. Isaacson, and B. A. White. 2004. Comparison of pulsed field gel electrophoresis and repetitive sequence polymerase chain reaction as genotyping methods for detection of genetic diversity and inferring transmission of *Salmonella*. Vet. Microbiol. 100:205-17.

37. Weijtens M. J., H. A. Urlings, and J. Van der Plas. 2000. Establishing a campylobacter-free pig population through a top-down approach. Lett. Appl. Microbiol. 30:479-484.

38. Wheeler, D. L., T. Barrett, D. A. Benson, S. H. Bryant, K. Canese, V. Chetvernin, D. M. Church, M. DiCuccio, R. Edgar, S. Federhen, L. Y. Geer, Y. Kapustin, O. Khovayko, D. Landsman, D. J. Lipman, T. L. Madden, D. R. Maglott, J. Ostell, V. Miller, K. D.

Pruitt, G. D. Schuler, E. Sequeira, S. T. Sherry, K. Sirotkin, A. Souvorov, G. Starchenko, R. L. Tatusov, T. A. Tatusova, L. Wagner, and E. Yaschenko. 2007. Database resources of the National Center for Biotechnology Information. Nucleic Acids Res. 35, D5-12.

Received 30 May 2017
Accepted 8 September 2017