ELSEVIER

Available online at www.sciencedirect.com

SCIENCE DIRECT

International Journal of Food Microbiology 105 (2005) 245–255

INTERNATIONAL JOURNAL OF Food Microbiology

www.elsevier.com/locate/ijfoodmicro

Development and application of oligonucleotide probes for in situ detection of thermotolerant *Campylobacter* in chicken faecal and liver samples

Michael W. Schmid [a], Angelika Lehner [b,*], Roger Stephan [b], Karl-Heinz Schleifer [c], Harald Meier [d]

[a] *GSF-Forschungszentrum für Umwelt und Gesundheit, Institut für Bodenökologie, Abteilung Rhizosphärenbiologie, Ingolstädter Landstr. 1, D-85764 Neuherberg, Germany*

[b] *Institut für Lebensmittelsicherheit und -hygiene, Universität Zürich, Winterthurerstrasse 272, CH-8057, Zürich, Switzerland*

[c] *Technische Universität München, Lehrstuhl für Mikrobiologie, Am Hochanger 4, D-85350 Freising, Germany*

[d] *Technische Universität München, Institut für Informatik, Lehrstuhl für Rechnertechnik und Rechnerorganisation/Parallelrechnerarchitektur, Angewandte Bioinformatik, Boltzmannstraße 3, D -85748 Garching bei München, Germany*

Received 10 May 2004; received in revised form 22 January 2005; accepted 19 April 2005

Abstract

Based on *Campylobacter* 16*S*- and 23*S*-rRNA sequence data oligonucleotide probes specific for thermotolerant campylobacters and for members of the genus *Campylobacter* have been developed. The 16*S*-rRNA-targeted probe CAMP653, recommended for a comprehensive detection of members of the genus *Campylobacter*, specifically detected all *Campylobacter* strains used in this study. Detection of thermotolerant species has been achieved by the 23*S*-rRNA-targeted probe CAJECO1427. Optimal hybridisation conditions have been derived for both probes from melting profiles of fluorescence-labelled probe-target hybrids recorded in fluorescence in situ hybridisation experiments (FISH).

The FISH assay was evaluated both by spiking poultry faecal samples with *Campylobacter jejuni* and by detecting *Campylobacter* in naturally colonized chickens. *C. jejuni* was reliably detected at levels of 10^6 cfu/g faeces after a 3- h enrichment step in Blood Preston Selective broth. Low level contaminations ($\leq 10^2$ cfu/g) were reliably identified after 24 h of enrichment. By screening cloacal swab samples obtained from birds from a poultry slaughterhouse, thermotolerant *Campylobacter* spp. were even detected without pre-enrichment.

Moreover, the in situ assay was applied on poultry liver samples. The probes allowed a direct, specific detection of thermotolerant *Campylobacter* in cryosectioned liver samples.

* Corresponding author. Tel.: +41 1 6358670; fax: +41 1 635 89 08.
E-mail address: lehnera@fsafety.unizh.ch (A. Lehner).
URL: http://www.ils.unizh.ch (A. Lehner).

0168-1605/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijfoodmicro.2005.04.012

Campylobacter spp. detection by FISH using highly specific probes looks promising to become a future monitoring system in a logistic poultry slaughter concept.
© 2005 Elsevier B.V. All rights reserved.

Keywords: In situ hybridisation; Thermotolerant *Campylobacter*; Chicken liver; Fluorescent probes; 16*S*-rRNA; 23*S*-rRNA; ARB

1. Introduction

Thermophilic *Campylobacter* species are recognised worldwide as an important cause of bacterial gastrointestinal human infections and are a major concern to the poultry industry due to high herd level prevalence (On, 1997; Frost et al., 1998; Altekruse et al., 1999; World Health Organization, 2000). They are efficient asymptomatic colonizers of poultry. If a flock is infected with *Campylobacter*, then the majority of the birds in that flock will be bacterial carriers (Berndtson et al., 1996a,b). Cross-contaminated foods caused by raw poultry meat during food preparation, unpasteurised milk and contaminated water are the most common sources for epidemic and sporadic foodborne cases (Altekruse et al., 1999; Pearson et al., 2000; Tauxe, 2002). In patients, *Campylobacter* are usually associated with self-limiting diarrhoea. However, *Campylobacter jejuni* and *Campylobacter coli* have also been implicated in extraintestinal diseases (Blaser, 1997).

Detection of *Campylobacter* is generally performed in a one-step cultural enrichment process under microaerophilic conditions at 42 °C and takes on an average of 4 to 5 days until the biochemical identification of a *Campylobacter* spp. suspicious colony is completed. Using the standard confirmation tests, species identification remains tedious (Weino et al., 2003). Furthermore, for *C. jejuni* a viable but nonculturable state is reported (Jones et al., 1991; Bovill and Mackey, 1997). This form seems mainly influenced by environmental stress conditions.

In the last years, the development and application of molecular techniques has provided alternatives for the detection, identification and characterization of foodborne organisms with a significantly shorter turnover time compared to culture-based methods. Different PCR based approaches have been applied for *Campylobacter* spp. in food and environmental samples (Giesendorf and Quint, 1995; Harmon et al., 1997; Manfreda et al., 2003; Rudi et al., 2004). Recently, Josefsen et al. (2004) evaluated through a collaborative trial a PCR-based method for the detection of thermotolerant campylobacters in spiked carcass rinse samples. However, most of the already published PCR-based methods have not included an internal amplification control. Moreover, when direct application of PCR in complex matrixes is performed, many inhibitory substances significantly influence the effectiveness on the activity of the *Taq* polymerase-based system (Abbaszadegan et al., 1993; Lawson et al., 1997).

Fluorescence in situ hybridisation (FISH) with rRNA-targeted oligonucleotide probes is an alternative rapid and specific method for identification of bacteria, which is less prone to inhibitory substances. Recently, Moreno et al. (2003) reported the application of this technique for specific detection of *Campylobacter* spp. in water and sewage. However, no information on the probe sequences has been found.

The objective of this study was to design new oligonucleotide probes for the specific detection of *Campylobacter* spp. as well as for the specific detection of the thermotolerant *Campylobacter* group and their application in a FISH assay for poultry faecal and liver samples.

2. Material and methods

2.1. Reference strains

12 *Campylobacter* spp. strains as well as 14 non-*Campylobacter* reference strains from selected species were included in the study for probe specificity tests (Table 1). The strains were grown on selective agar plates under appropriate conditions. Grown colonies were swabbed from the plate using 1 ml PBS (130 mM sodium chloride, 10 mM sodium phosphate, pH 7.2) and fixed with three volumes of 4% paraformaldehyde according to the protocol described by Amann et al. (1995).

Table 1
Strains of bacteria used in this study and their reaction with the probes CAMP653 and CAJECO1427 after whole cell hybridization

Species	Strain	CAMP653	CAJECO1427
Arcobacter butzleri	NCTC 12713	–	–
Arcobacter skirrowii	NCTC 12481	–	–
Campylobacter coli	NCTC 11366^T	++	++
Campylobacter fetus	NCTC 10842	+	–
Campylobacter helveticus	NCTC 12470	+	+
Campylobacter hyointestinalis	NCTC 11608^T	+	+
Campylobacter jejuni	NCTC 11168	+	++
Campylobacter jejuni	NCTC 12506	+	++
Campylobacter lari	NCTC 11352	+	++
Campylobacter sp.	Water isolate	++	++
Campylobacter sp.	Clinical isolate	++	++
Campylobacter sp.	Clinical isolate	++	++
Campylobacter upsaliensis	NCTC 11845	++	++
Campylobacter mucosalis	LMG6448^T	+	–
Clostridium perfringens	ATCC 13124	–	–
Enterococcus faecalis	DSM 20478	–	–
Enterococcus faecium	DSM 20477	–	–
Escherichia coli O:157	NCTC 12900	–	–
Helicobacter pylori	NCTC 11637	+/–	–
Klebsiella pneumoniae	DSMZ 30104	–	–
Listeria monocytogenes	DSMZ 12464	–	–
Pseudomonas aeruginosa	ATCC 10145	–	–
Pseudomonas aeruginosa	DSMZ 50071	–	–
Salmonella Typhimurium	ATCC13311	–	–
Wolinella succinogenes	NCTC 11488	–	–
Yersinia enterocolotica	DSMZ 4780	–	–

(–) no signal; (+), signal; (++) strong signal; (+/–) signal at 35% formamide, no signal at 50% formamide in the hybridization buffer.

2.2. Spiked poultry faeces samples

10 g chicken faeces samples were first sterilised by autoclaving (20 min, 121 °C) and then rehydrated with sterile water to substitute the loss of weight. For spiking, an overnight culture (1.4×10^9 cfu/ml) of *C. jejuni* (NCTC 11168) was diluted in tenfold steps and 200 μl of the culture dilutions was added to 2 mg aliquots of the sterilized faeces. 200 μl of each sample was immediately fixed according to the protocol by Amann et al. (1995) and resuspended in 200 μl PBS/$EtOH_{abs}$ (1:1 (v/v)). Furthermore, 1 ml of each sample was transferred into 5 ml Nutrient Selective Broth (NB No2 OXOID, supplemented with cephoperazone and FBP, OXOID, UK) and incubated at 37 °C under microaerophilic conditions. After 3, 6, 9, 24 and 48 h of incubation 400 μl aliquot was fixed and resuspended.

To study the influence of the high level of background flora, 1 g aliquot of non-autoclaved faeces was diluted in 1.9 ml PBS and also spiked with 100 μl from serial dilutions steps of the *C. jejuni* overnight culture. 200 μl of each sample was then washed with PBS and fixed immediately. The rest of each sample was transferred into 5 ml of Nutrient Selective Broth (NB No2 OXOID, supplemented with cephoperazone and FBP, OXOID, UK) and incubated. Aliquots were fixed after 24 h of incubation and resuspended as described before.

2.3. Chicken liver samples

Fresh chicken livers from 8 animals were purchased from retail. 1 × 1 cm pieces of each liver were embedded in cryomedium (Microm, Walldorf, Germany) and frozen at −80 °C for at least 2 h. Sections ranging from 5 to 10 μm were obtained using a cooled microtome (Microm, Walldorf, Germany). The slices were placed on Teflon-coated glass slides (10 reaction fields per slide; Marienfeld, Bad Mergentheim, Germany) and the material was fixed on the slide by incubation with 20 μl of 3% PFA

solution (see preparation above) per well for 20 min at 4 °C. Slides were rinsed once in PBS (130 mM sodium chloride, 10 mM sodium phosphate, pH 7.2) and immersed for 3 min each in 50%, 80% and 100% EtOH.

2.4. Probe design, synthesis and labelling

Probe design was performed by computer aided comparative analysis of ssu- and lsu-rRNA sequence datasets using the PROBE_DESIGN tool included in the software package ARB (Ludwig et al., 2004). The specificities of the probes were evaluated using PROBE_MATCH of the ARB software. BLAST searches were performed against the non-redundant database (nr) of EMBL/GENBANK. Probes were synthesized and labelled with the fluorochromes FLUOS, Cy3 or Cy5 by Thermo Hybaid (Division Interactiva, Ulm, Germany).

2.5. Fluorescence in situ hybridisation

Hybridisations were performed on Teflon-coated glass slides with 6 or 10 wells (Marienfeld, Bad Mergentheim, Germany) for independent positioning of the samples. Aliquots of reference cells were spotted on single wells, dried at 46 °C and dehydrated using an ascending ethanol series (5 min each, 50%, 80% and 100% ethanol). Hybridisations were carried out for at least 1.5 h at 46 °C in 10 μl hybridisation buffer containing 0.9 M sodium chloride (pH 8.0), 0.01% (w/v) SDS, 10 mM Tris–HCl (pH 8.0), various amounts of deionized formamide, and either 30 ng of Cy3 and Cy5-labelled or 50 ng of FLUOS-labelled probes. The fluorescence signal of the bacteria-specific probe EUB338-I, -II, -III-FLUOS (Amann et al., 1990; Daims et al., 1999), in short EUB338mix-FLUOS, served as a reference value for optimization experiments. After hybridisation had taken place in a humid chamber, slides were rinsed with pre-warmed washing buffer and washed for 20 min at 48 °C in a water bath as previously described (Neef et al., 1998). The washing buffer contained 20 mM Tris–HCl (pH 8.0), 0.01% (w/v) SDS, and sodium chloride from 0.056 M to 0.9 M depending on the stringency according to the hybridisation buffer. To remove salts, the slides were rinsed with deionized water. After air drying the slides, the samples were embedded in Citifluor AF-1 (Citifluor Ltd., Canterburry, UK) to undergo bleaching effects and sealed with a cover slip.

2.6. Fluorescence and quantification of fluorescence values

Fluorescence intensities of the hybrids were recorded using a charged coupled device (CCD) CAMERA (CF—15/2; Kappa, Gleichen, Germany) attached to the straight-through port of the epifluorescence microscope. The interline CCD chip with an integrated mosaic filter consisted of 681 $(H) \times 582$ (V) pixels with a pixel size of 9.6 μm $(H) \times 8.4$ μm (V). The analogue output-signal of the camera was delivered to a color framegrabber (Imaging Technology, Bedford, MA, USA) for 8-bit image processing using the Optimas software (BioScan, Edmonds, WA, USA). Camera parameters were held at fixed settings for all measurements. For each picture a threshold was adjusted to separate the probe conferred fluorescence from background noise and the corresponding areas were marked. The luminescence signals belonging to these areas were automatically extracted from the image and further processed using a statistic software. For the different hybridisation conditions or probe types 50–100 cells were analyzed.

2.7. Epifluorescence and confocal laser scanning microscopy

Epifluorescence microscopy was performed with an Axioplan microscope (Axioplan-2, Zeiss, Oberkochen, Germany) equipped with high quality filter sets for FLUOS and Cy3 excitation. For the documentation of the hybridisation results a confocal laser scanning microscope (LSM-510, Zeiss) was used in addition. An argon ion laser supplied a wavelength of 488 nm for excitation of FLUOS and two helium–neon lasers provided excitation wavelengths of 543 nm and 633 nm for Cy3 and Cy5, respectively.

2.8. Oligonucleotide probes and probe hybridisation behaviour

The 16*S*-rRNA targeting probe (binding position 653–670) CAMP653 (5′–CTGCCTCTCCCTYACTCT–3′) for detection of members of the genus *Campy-*

lobacter and the 23*S*-rRNA targeting probe (binding position 1427–1444) CAJECO1427 (5′–AGCCCTA-AGCGTCCTTCC–3′) for the detection of thermotolerant *Campylobacter* species were designed by applying the ARB tool PROBE DESIGN on special data structures (PT-Servers) derived from the ssu-rRNA database "ssu_jan03.arb" and the lsu-rRNA database "3pubmrz99.arb" (ARB-Homepage, www.arb-home.de). Probe match results of the newly designed probes CAMP653 and CAJECO1427 for *Campylobacter* spp. as well as selected non-target species are given in Figs. 1 and 2, respectively. *Campylobacter mucosalis* has sequence data that are quite similar to the perfect target sequences of probe CAJECO1427, containing only two mismatches that are located quite close to the border. Therefore *C. mucosalis* was chosen as negative reference in the further hybridisation experiments for determining the conditions for specific application of the probes (Table 2).

The mean fluorescence signals of the probes CAMP653-FLUOS or CAJECO1427-Cy3 in fixed cells of *C. jejuni* and *C. mucosalis* at different stringencies compared to the fluorescence signal of the bacteria-specific probe EUB338mix-FLUOS are shown in Fig. 3. In cells of *C. jejuni* and *C. mucosalis* the fluorescent signals of probe EUB338mix-FLUOS was brightest when applied at 30% formamide. Cells of *C. mucosalis* show even brighter fluorescence (for 30% formamide in the hybridisation buffer) than cells

```
Probe sequence                    5´-CTGCCTCTCCCTYACTCT-3´

Target binding site               5´-AGAGURAGGGAGAGGCAG-3´

Campylobacter curvus             UCU-==================-AUG
Campylobacter concisus           UCU-==================-AUG
Campylobacter mucosalis          UCU-==================-AUG
Campylobacter fetus              UCU-==================-AUG
Campylobacter fetus subsp fetus  UCU-==================-AUG
Campylobacter hyointestinalis    UCU-==================-AUG
Campylobacter lanienae           UCU-==================-AUG
Campylobacter hyoilei            UCU-==================-AUG
Campylobacter jejuni subsp jejuni UCU-==================-AUG
Campylobacter jejuni             UCU-==================-AUG
Campylobacter helveticus         UCU-==================-AUG
Campylobacter upsaliensis        UCU-==================-AUG
Campylobacter coli               UCU-==================-AUG
Campylobacter gracilis           UCU-==================-AUG
Campylobacter sputorum           UCU-==================-AUG
Campylobacter rectus             UCU-==================-AUG
Campylobacter showae             UCU-==================-AUG
Campylobacter hominis            UCU-======g===========-AUG
Campylobacter lari               UCU-=====gg===========-AUG
Campylobacter curvus             UCU-===============u==-AUG
Thermanaerovibrio velox          UCU-====N=N========aNA-GCG
Thermoactinomyces vulgaris       GCU-N=====NN=======GgA-GCG
Helicobacter bovis               UCU-g=====U===========-GUG
Helicobacter acinonyx            UCU-g=====U========N==-GUG
Campylobacter gracilis           UCU-=====A============-AUG
Campylobacter sp oral clone      UCU-=====A============-AUG
Campylobacter sputorum           UCU-=====A============-AUG
Campylobacter rectus             UCU-=====A============-AUG
Campylobacter showae             UCU-=====A============-AUG
Helicobacter muridarum           UCU-======U========u==-GUG
Helicobacter cinaedi             UCU-======U========u==-GUG
Helicobacter fennelliae          UCU-======U========u==-GUG
Helicobacter felis               CCU-======U========u==-GUG
Helicobacter heilmannii          CCU-======U========u==-GUG
Helicobacter pylori              UCU-======U========u==-GUG
```

Fig. 1. Probe match of the newly designed probe CAMP653. Binding position 653–670 at the 16*S*-rRNA according to Brosius et al. (1981).

```
Probe sequence                 5´-AGCCCTAAGCGTCCTTCC-3´

Target binding site            5´-GGAAGGACGCUUAGGGCU-3´


Campylobacter jejuni          GAU-==================-AAG
Campylobacter coli            GAU-==================-AAG
Campylobacter hyoilei         GAU-==================-AAG
Campylobacter sp.             GAU-==================-AAG
Campylobacter lari            GAU-==================-AAG
Campylobacter upsaliensis     GAU-==================-AAG
Campylobacter mucosalis       GAU-==============A=u=-AAA
Campylobacter concisus        GAU-==============A=u=-AAG
Pediococcus dextrinicus       GAU-===g======A=======-AAG
Prionace glauca               CUC-==u====g=====ug==u-CUU
Helicobacter pylori           GAU-==gg======A=====u=-AAG
Wolinella succinogenes        GAC-==gg======A=====u=-AAA
Mit. Acanthamoeba castellanii AUG-g========a======au-CGA
Acanthamoeba castellanii      AUG-g========a======au-CGA
Acanthamoeba castellanii      AUG-g========a======au-CGA
Sulfolobus solfataricus       GGU-===g==C==NN=====G=-UCU
Salvelinus namaycush          GUG-=====ggg======gg==-ACA
Uronema belkae                GCC-g=Ga===========g==-GAA
Bacillus halodurans           UGC-============C==A==-UUU
Alvinella pompejana           UNG-g=====UN=N=====C=u-GAU
```

Fig. 2. Probe match of the newly designed probe CAJECO1427. Binding position 1427–1444 at the 23*S*-rRNA according to Brosius et al. (1981).

of *C. jejuni* under the same hybridisation conditions. For the probe CAJECO1427-CY3, however, the highest signal intensities were measured with *C. jejuni* hybridized with 20% and 35% formamide in the hybridisation buffer, whereas no signal has been detected in cells of *C. mucosalis* hybridized even at less stringent conditions (0, 5%, 10% formamide, respectively) due to three weak and three strong mismatches in the probe specific target sequence.

Probe CAMP653-FLUOS showed different hybridisation behaviour. At each formamide concentration the signal was less intense than the respective signal of the probes EUB338mix and CAJECO1427.

3. Results

3.1. Validation and optimization of the FISH system on target and non-target strains

Results of the hybridisation experiments are summarized in Table 1. The 16*S*-rRNA-targeted probe CAMP653 detected all strains of the genus *Campylobacter* included in this study. Probe CAJECO1427 specifically hybridised with strains of *C. coli*, *C. jejuni* and other most important thermotolerant species of the genus *Campylobacter*. Hybridisation with the genus-specific probe CAMP653 on *Helicobacter*

Table 2
Sequence, specificity, binding positions and references for all probes used in this study

Probe	Binding position, *E. coli* (Brosius et al., 1981)	Specificity	Reference	Probe sequence [5′–3′]
EUB338-I[a]	16*S*-rRNA, 338–355	Most *Bacteria* except * and **	Amann et al. (1990)	GCT-GCC-TCC-CGT-AGG-AGT
EUB338-II[a]	16*S*-rRNA, 338–355	*Planctomycetales	Daims et al. (1999)	GCA-GCC-ACC-CGT-AGG-TGT
EUB338-III[a]	16*S*-rRNS, 338–355	**Verrucomicrobiales	Daims et al. (1999)	GCA-GCC-ACC-CGT-AGG-TGT
CAMP653	16*S*-rRNA, 653–670	See Fig. 1	This study	CTG-CCT-CTC-CCT-YAC-TCT
CAJECO1427	23*S*-rRNA, 1421–1438	See Fig. 2	This study	AGC-CCT-AAG-CGT-CCT-TCC

[a] EUB338-I, -II and -III = EUB338mix.

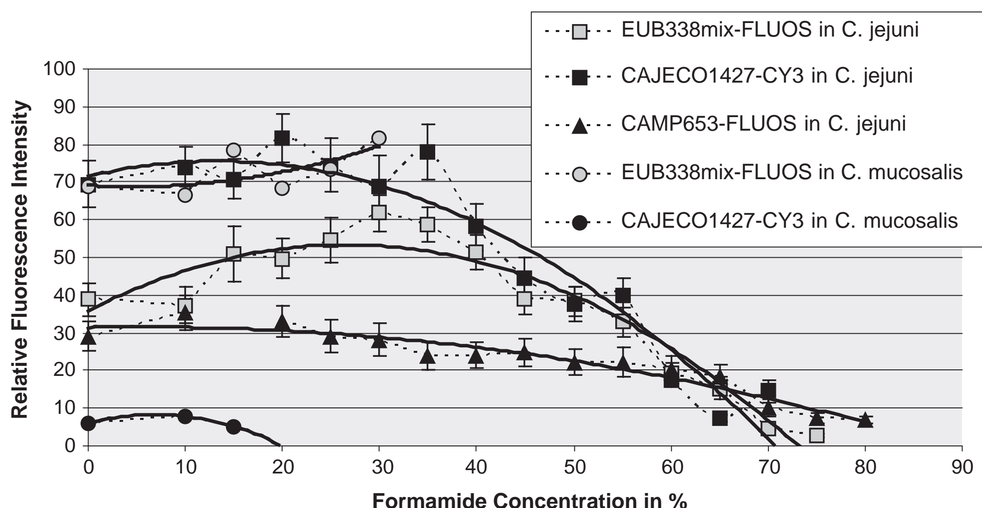

Fig. 3. In situ melting curves of the probes CAMP653, CAJECO1427 and EUB338. The fluorescence signals were measured in whole fixed *Campylobacter* cells hybridized at a range of 0 (low stringency) to 80% (high stringency) formamide in hybridization buffer.

pylori strains showed a signal, when 35% formamide was used in the hybridisation buffer. However, no signal could be observed, when 50% formamide was applied in the hybridisation buffer. For further experiments, a concentration of 35% formamide was applied in the hybridisation buffer, when only CAJECO1427-Cy3 was used, and 45–50% when dual hybridisations with probe CAMP653-FLUOS were performed in faeces and bowel samples.

3.2. Detection of Campylobacter by FISH in spiked faecal samples

C. jejuni was reliably detected in spiked sterilized faecal samples at levels of 10^6 cfu/g after a 3-h enrichment step in Blood Preston Selective broth. Low level contaminations ($<10^2$ cfu/g) were reliably identified after 24 h of enrichment. Contamination levels higher than 10^6 cfu/g faeces could be detected by in situ analysis without prior cultivation (Tables 3A and 3B).

Furthermore, in situ hybridisation using the probes EUB338mix-FLUOS and CAJECO1427-Cy3 identified *C. jejuni* cells unambiguously within non-autoclaved chicken faeces samples spiked with 10^2 cfu/g after 24 h of enrichment. Representative pictures summarising these results are shown in Fig. 4A and B.

Preliminary experiments on screening cloacal swab samples obtained from birds from a poultry slaughterhouse with the cultural and FISH method, thermotolerant *Campylobacter* spp. could even be detected without pre-enrichment, indicating high level shedding of *Campylobacter* in poultry.

Table 3A
Results of in situ experiments (CAJECO1427) on artificially contaminated faeces samples as a function of contamination dose and the time period of selective enrichment

Incubation time (h)	Inoculum					
0	10^8 (+)	10^6 (+/−)[a]	10^5 (−)	10^4 (−)	10^3 (−)	10^2 (−)
3	10^8 (+)	10^6 (+)	10^5 (+/−)[a]	10^4 (−)	10^3 (−)	10^2 (−)
6	10^8 (+)	10^6 (+)	10^5 (+)	10^4 (+/−)[a]	10^3 (−)	10^2 (−)
9	10^8 (+)	10^6 (+)	10^5 (+)	10^4 (+)	10^3 (+)	10^2 (+/−)[a]
24	10^8 (+)	10^6 (+)	10^5 (+)	10^4 (+)	10^3 (+)	10^2 (+)

[a] Unambiguous sample (+/−) when microscopic fields (magnification: 1000×) with "*Campylobacter*-positive" cells were rarely found and contained less than 10 cells.

Table 3B
Cell numbers of *Campylobacter jejuni* as detected by FISH using CAJECO1427-Cy3 in artificially contaminated faeces

Selective enrichment: Time (h)	*C. jejuni* cell counts [triplicates (contamination dose)]	*C. jejuni* cell counts (contamination dose)	*C. jejuni* cell counts (contamination dose)
T: 0	58/36/61 (10^8 cells/g faeces)		
T: 3	26/24/17 (10^6 cells/g faeces)		
T: 6	40/24/36 (10^6 cells/g faeces)	33/29/38 (10^5 cells/g faeces)	10/12/8 (10^4 cells/g faeces)
T: 9	42/34/46 (10^4 cells/g faeces)	19/17/14 (10^3 cells/g faeces)	14/11/5 (10^2 cell/g faeces)
T: 24	800/1023/1290 (10^3 cell/g faeces)	530/454/420 (10^2 cell/g faeces)	

Counts from a microscopic area of 7200 μm^2 (magnification: 1000×).

3.3. In situ detection of thermotolerant Campylobacter in liver samples

In situ analysis revealed the presence of thermotolerant *Campylobacter* in all of the eight samples, appearing as fluorescent clusters within the large liver cells. A representative picture summarizing theses results is shown in Fig. 4C and D.

For confirmation reasons, fresh liver samples were also homogenized and plated on *Campylobacter* selective agar. One colony from morphologically typical colonies was picked and used for PCR amplification and sequencing of 16*S*-rRNA and 23*S*-rRNA gene fragments. All of the sequences analyzed could phylogenetically be affiliated to *Campylobacter* spp., and in situ hybridisation of

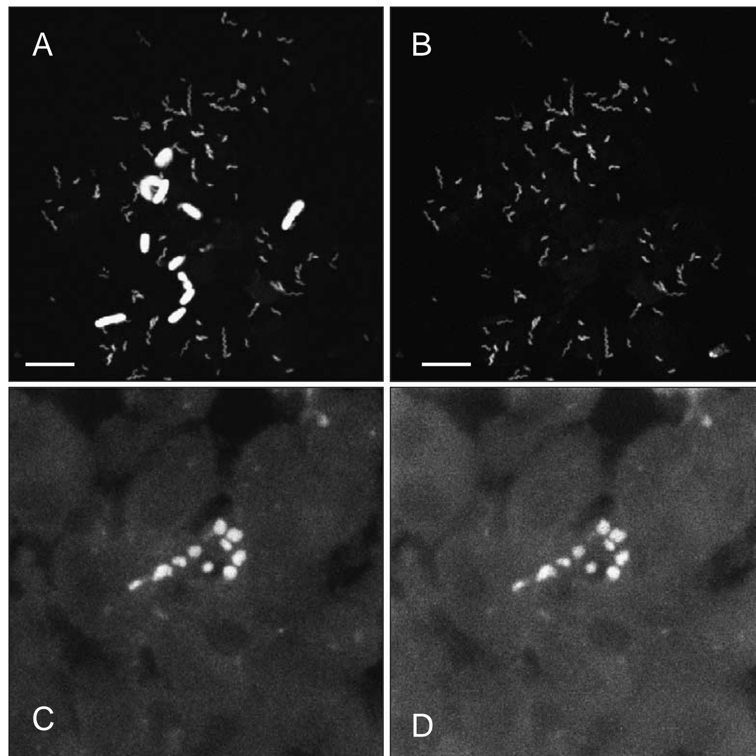

Fig. 4. In situ hybridization of natural chicken faeces spiked with 10^2 cfu *C. jejuni* after 24h of enrichment in selective Nutrient Broth. *C. jejuni* and cells of the natural flora are detected by EUB338mix-FLUOS (A) and cells of *C. jejuni* specifically detected by CAJECO1427-Cy3 (B). Cryosections of chicken liver after FISH hybridization. Fluorescence signal derived from the probes (C) CAMP653-Cy3 and (D) CAJECO1427-FLUOS.

fixed morphologically typical colonies resulted in positive hybridisation signals using the probes CAMP653 and CAJECO1427 (data not shown).

4. Discussion

Fluorescence in situ hybridisation (FISH) with rRNA-targeted oligonucleotide probes is widely used in microbial ecology (Amann et al., 1995; Amann and Schleifer, 2001). The method has also been successfully applied for the specific and reliable detection of enterococci in water samples (Meier et al., 1997) as well as for the identification of pathogenic bacteria in complex matrixes such as blood and tissue samples (Kempf et al., 2000; Trebesius et al., 2000). Moreover, several FISH-based detection kits are already commercially available for the identification of e.g. beer spoiling bacteria or pathogenic organisms in water and food (www.vermicon.com). The main advantage of the FISH method over PCR-based systems is the fact that it is more stable against influences by matrix inhibitors, as this system is based on specific duplex formation of complementary nucleic acid strands and fluorescence emission, and works independently from any enzymatic reaction (Jones et al., 1991; Moreno et al., 2001).

In this study we present for the first time sequence data for specific probes for *Campylobacter* spp. and the thermotolerant *Campylobacter* group, respectively, the most prevalent foodborne pathogen worldwide. Applying the fluorescent derivatives of these probes for FISH-based detection of *Campylobacter* exhibit a major improvement. While detection and correct identification of *Campylobacter* by selective cultivation is not always unambiguous as the success depend strongly on the specificity and sensitivity of the selective media used (Baylis et al., 2000), an application of FISH probes provides definite results due to the specific nature of the probes (Fig. 4A and B).

Campylobacter spp. are carried asymptomatically in high numbers in the intestinal tract of poultry (Berndtson et al., 1996a,b). Moreover, if a flock is infected with *Campylobacter*, then the majority of the birds in that flock will be bacterial carriers. These two aspects are a major hygienic problem in the poultry slaughtering process. Rapid detection of *Campylobacter* in faecal samples for identification infected flocks prior to slaughter, in order to provide the consumers with *Campylobacter*-free chickens, would therefore improve food safety. The implementation of the so-called "strategic slaughter", where infected flocks are slaughtered at the end of the day, seems to have contributed to the recent significant decline of human campylobacteriosis in Denmark (www.dzc.dk).

Therefore, the poultry industry increasingly depends on robust rapid tests for screening faecal or cloacal swab samples on flock level 1 or 2 days before slaughtering, which deliver *Campylobacter* results within a short time and which allow to release flocks for slaughter dependent on such results. By application the designed and evaluated probes within a FISH system in poultry faecal samples we were able to reliably detect *C. jejuni* in spiked samples at levels of higher than 10^6 cfu/g after 3 h and low level contaminations ($\leq 10^2$ cfu/g) after a 24-h enrichment step. Furthermore, as preliminary data on cloacal swab samples obtained from birds from a poultry slaughterhouse showed, in situ detection of thermotolerant *Campylobacter* spp. was possible without pre-enrichment. However, this finding needs further evaluation.

To conclude, fluorescent in situ hybridization using highly specific probes can be an alternative time saving, reliable and cultivation-independent technique for the detection and identification of *Campylobacter* in chicken flocks. This technique looks promising to be used in the future, maybe in a commercial kit version as a "close to real time" monitoring system in a logistic slaughter concept.

References

Abbaszadegan, M., Huber, M.S., Gerba, Ch.P., Pepper, I.L., 1993. Detection of enteroviruses in groundwater with the polymerase chain reaction. Applied and Environmental Microbiology 59, 1318–1324.

Altekruse, S.F., Stern, N.J., Fields, P.I., Swerdlow, D.L., 1999. *Campylobacter jejuni*—an emerging foodborne pathogen. Emerging Infectious Diseases 5, 28–35.

Amann, R., Schleifer, K.H., 2001. Nucleic acid probes and their application in environmental microbiology. In: Boone, D.R., Castenholz, R.W., Garrity, G.M. (Eds.), Bergey's Manual of

Systematic Bacteriology, (2nd edition)Springer Press, New York, pp. 67–82.

Amann, R.I., Krumholz, L., Stahl, D.A., 1990. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic and environmental studies in microbiology. Journal of Bacteriology 172, 762–770.

Amann, R.I., Ludwig, W., Schleifer, K.H., 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiological Reviews 59, 143–169.

Baylis, C.L., MacPhee, S., Martin, K.W., Humphrey, T.J., Betts, R.P., 2000. Comparison of three enrichment media for the isolation of *Campylobacter* spp. from foods. Journal of Applied Microbiology 89, 884–891.

Berndtson, E., Danielsson-Tham, M.L., Engvall, A., 1996a. *Campylobacter* incidence on a chicken farm and the spread of *Campylobacter* during the slaughter process. International Journal of Food Microbiolgy 32, 35–47.

Berndtson, E., Emanuelson, U., Engvall, A., Danielsson-Tham, M.L., 1996b. A 1-year epidemiological study of *Campylobacter* in 18 Swedish chicken farms. Veterinary Microbiology 26, 167–185.

Blaser, M.J., 1997. Epidemiologic and clinical features of *Campylobacter jejuni* infections. Journal of Infectious Diseases 176 (2), 103–105.

Bovill, R.A., Mackey, B.M., 1997. Resuscitation of "non-culturable" cells from aged cultures of *Campylobacter jejuni*. Microbiology 143, 1575–1581.

Brosius, J., Dull, T.J., Sleeter, D.D., Noller, H.F., 1981. Gene organization and primary structure of a ribosomal RNA operon from *E. coli*. Journal of Molecular Biology 148, 107–127.

Daims, H., Brühl, A., Amann, R.I., Schleifer, K.H., Wagner, M., 1999. The domain-specific probe EUB338 is insufficient for the detection of all *bacteria*: development and evaluation of a more comprehensive probe set. Systematic and Applied Microbiology 22, 434–444.

Frost, J.A., Oza, A.N., Thwaites, R.T., Rowe, B., 1998. Serotyping scheme for *Campylobacter jejuni* and *Campylobacter coli* based on direct agglutination of heat-stable antigens. Journal of Clinical Microbiology 36, 335–339.

Giesendorf, B.A., Quint, W.G., 1995. Detection and identification of *Campylobacter* spp. using the polymerase chain reaction. Cellular and Molecular Biology 41, 625–638.

Harmon, K., Ransom, G., Wesley, I., 1997. Differentiation of *Campylobacter jejuni* and *Campylobacter coli* by multiplex polymerase chain reaction. Molecular and Cellular Probes 11, 195–200.

Jones, D.M., Sutcliffe, E.F., Curry, A., 1991. Recovery of viable but non-culturable *Campylobacter jejuni*. Journal of General Microbiology 137, 2477–2482.

Josefsen, M.H., Cook, N., D'Agostino, M., Hansen, F., Wagner, M., Demnerova, K., Heuvelink, A.E., Tassios, P.T., Lindmark, H., Kmet, V., Barbanera, M., Fach, P., Loncarevic, S., Hoorfar, J., 2004. Validation of a PCR-based method for detection of food-borne thermotolerant campylobacters in a multicenter collaborative trial. Applied and Environmental Microbiology 70, 4379–4383.

Kempf, V.A., Trebesius, K.H., Autenrieth, I.B., 2000. Fluorescent in situ hybridization allows rapid identification of microorganisms in blood cultures. Journal of Clinical Microbiology 38, 830–838.

Lawson, A.J., Linton, D., Stanley, J., Owen, R.J., 1997. Polymerase chain reaction detection and speciation of *Campylobacter upsaliensis* and *C. helveticus* in human faeces and comparison with culture techniques. Journal of Applied Microbiology 83, 375–380.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Buchner, A., Lai, T., Steppi, S., Jobb, G., Förster, W., Brettske, I., Gerber, S., Ginhart, A.W., Gross, O., Grumann, S., Hermann, S., Jost, R., König, A., Liss, T., Lüßmann, R., May, M., Nonhoff, B., Reichel, B., Strehlow, R., Stamatakis, A.P., Stuckmann, N., Vilbig, A., Lenke, M., Ludwig, T., Bode, A., Schleifer, K.H., 2004. ARB: a software environment for sequence data. Nucleic Acids Research 32, 1363–1371.

Manfreda, G., De Cesare, A., Bondioli, V., Franchini, A., 2003. Comparison of the BAX System with a multiplex PCR method for simultaneous detection and identification of *Campylobacter jejuni* and *Campylobacter coli* in environmental samples. International Journal of Food Microbiology 87, 271–278.

Meier, H., Koob, C., Ludwig, W., Amann, R., Frahm, E., Obst, U., Hoffmann, S., Schleifer, K.H., 1997. Detection of *Enterococci* on group and species level with rRNA targeted DNA probes for hygienical drinking water control. Water Science and Technology 35, 437–444.

Moreno, Y., Hernandez, M., Ferrus, A.A., Alonso, J.L., Botella, S., Montes, R., Hernandez, J., 2001. Direct detection of thermotolerant campylobacters in chicken products by PCR and in situ hybridization. Research in Microbiology 152, 577–582.

Moreno, Y., Botella, S., Alonso, J.L., Ferrus, M.A., Hernandez, M., Hernandez, J., 2003. Specific detection of *Arcobacter* and *Campylobacter* strains in water and sewage by PCR and in situ hybridization. Applied and Environmental Microbiology 69, 1181–1186.

Neef, A., Amann, R., Schlessner, H., Schleifer, K.H., 1998. Monitoring a widespread bacterial group: in situ detection of planctomycetes with 16*S*-rRNA-targeted probes. Microbiology 144, 3257–3266.

On, S., 1997. Identification methods for *Campylobacter*, *Helicobacter* and related organisms. Clinical Microbiology Reviews 9, 405–422.

Pearson, A.D., Greenwood, M.H., Donaldson, J., Healing, T.D., Jones, D.M., Shahamat, M., Feltham, R.K., Colwell, R.R., 2000. Continuous source outbreak of campylobacteriosis traced to chicken. Journal of Food Protection 63, 309–314.

Rudi, K., Hoidal, H.K., Katla, T., Johansen, B.K., Nordal, J., Jakobsen, K.S., 2004. Direct real-time PCR quantification of *Campylobacter jejuni* in chicken faecal and cecal samples by integrated cell concentration and DNA purification. Applied and Environmental Microbiology 70, 790–797.

Tauxe, R.V., 2002. Emerging foodborne pathogens. International Journal of Food Microbiology 78, 31–41.

Trebesius, K.-H, Panthel, K., Strobel, S., Vogt, K., Faller, G., Kirchner, T., Kist, M., Heesemann, J., Haas, R., 2000. Rapid and specific detection of *Helicobacter pylori* macrolide resis-

tance in gastric tissue by fluorescent in situ hybridisation. Gut 46, 608–614.

Weino, M., Bang, D.D., Lund, M., Nordentoft, S., Andersen, J.S., Pedersen, K., Madsen, M., 2003. Identification of *Campylobacter* isolated from Danish broilers by phenotypic tests and species-specific PCR assays. Journal of Applied Microbiology 95, 649–655.

World Health Organization, 2000. Factsheet No 255, Website of the WHO, http://www.who.int/mediacentre/factsheets/fs255/en, visited 2004-02-08.