JOURNAL OF CLINICAL MICROBIOLOGY, June 2008, p. 2133–2136 Vol. 46, No. 6
0095-1137/08/$08.00+0 doi:10.1128/JCM.01512-07

Identification of Thermotolerant *Campylobacter* Species by Fluorescence In Situ Hybridization[▽]

Sven Poppert,[1]* Michaela Haas,[1] Tatjana Yildiz,[1] Thomas Alter,[2] Edda Bartel,[2] Ursula Fricke,[1] and Andreas Essig[1]

Institute of Medical Microbiology and Hygiene, University of Ulm, Ulm, Germany,[1] and Federal Institute for Risk Assessment, Berlin, Germany[2]

Received 28 July 2007/Returned for modification 26 November 2007/Accepted 21 March 2008

Thermotolerant *Campylobacter* spp. (*Campylobacter jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis*) are leading causes of food-borne diarrhea in humans. In this study, the usefulness of fluorescence in situ hybridization (FISH) for the identification of *Campylobacter* isolates was investigated. A hierarchical FISH probe set that included six group-, genus-, and species-specific probes was developed and evaluated with 12 reference strains and 94 clinical isolates of *Campylobacter*, *Arcobacter*, and *Helicobacter*. FISH correctly identified all isolates to the genus level and detected all thermotolerant *Campylobacter* isolates. The assay showed high degrees of sensitivity for the identification of *C. jejuni* (90%), *C. coli* (97%), *C. lari* (81%), and *C. upsaliensis* (100%) to the species level.

Thermotolerant *Campylobacter* spp. (*Campylobacter jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis*) are leading causes of food-borne human gastroenteritis and the corresponding late-onset complications, such as reactive arthritis and Guillain-Barré syndrome (5). The phenotypic identification of *Campylobacter* spp. is complicated and of limited reliability (2, 5, 8, 11, 12). The identification of *C. lari* and *C. upsaliensis* to the species level and the discrimination of the close relative *Arcobacter* from *Campylobacter* are especially problematic, leading to uncertainty about the true clinical relevance of these organisms (2, 5, 8, 11, 12, 24). Various molecular methods have therefore been proposed as alternative diagnostic methods (5–7, 11–13, 20, 27). Among these, fluorescence in situ hybridization (FISH) has been described for the identification of *Campylobacter* (15, 25) and its relatives, *Helicobacter* and *Arcobacter* (3, 15, 16, 26), in environmental samples and chicken products. FISH is a microscopic method that uses fluorescently labeled oligonucleotide DNA probes that bind specifically to unique target sites on ribosomal RNA (10, 18, 23). The advantages of FISH are its simple methodology, high speed, low cost, and minimal equipment requirements (only a fluorescent microscope is needed) (10, 18, 23). The aim of this study was to establish and evaluate a FISH assay for the identification of thermotolerant *Campylobacter* in a clinical setting. A hierarchical set of six FISH probes (Table 1) was designed with ARB software (http://www.arb-home.de). One probe covers all *Campylobacter* and its relatives, *Arcobacter* and *Helicobacter* (the HelCArc probe). One group-specific probe targets the four thermotolerant *Campylobacter* spp. (the Catherm probe). Species-specific probes were designed for *C. jejuni* (the Cajej probe), *C. upsaliensis* (the Cup probe), and *C. lari* (the Clar1 and Clar2 probes). A combination of two probes was implemented for *C. lari*, since it was not possible to cover this heterogeneous species with a single probe. We did not succeed in designing a probe for *C. coli* with sufficient sensitivity.

Probes were directly 5′ labeled with the fluorescent dye Cy3 (red) or 6-carboxyfluorescein (FAM; green) (Thermo, Ulm, Germany). Hybridization was performed as described previ-

TABLE 1. Oligonucleotide probes

Probe	Target (position)	Target organism(s)	Sequence (5′–3′)	Reference or source
HelCArc	23S rRNA (1760)	*Campylobacter* spp., *Helicobacter* spp., and *Arcobacter* spp.	AAC AGT CGG GAG GGA CTC	This study
Catherm	23S rRNA (1419)	Thermotolerant *Campylobacter* spp	GCC CTA AGC GTC CTT CCA	This study
Cajej	23S rRNA (1693)	*C. jejuni*	AGC TAA CCA CAC CTT ATA CCG	This study
Clar 1[a]	16S rRNA (622)	*C. lari*	TCC CAA GCA GTT CAA CGG T	This study
Clar 2[a]	16S rRNA (1126)	*C. lari*	GAA GTG TTA GCA ACT AAA T	This study
Cup	16S rRNA (1695)	*C. upsaliensis*	CTC TAC AGA ATT TGT TGG AT	This study
EUB	16S rRNA (338)	Bacterial kingdom	GCT GCC TCC CGT AGG AGT	1

[a] The two *C. lari*-specific probes are used simultaneously.

* Corresponding author. Present address: Bernhard Nocht Institute for Tropical Medicine (BNI), Bernhard-Nocht-Strasse 74, Hamburg D-20359, Germany. Phone: 40-40-42818-0. Fax: 49-40-42818-400. E-mail: poppert@bni-hamburg.de.

▽ Published ahead of print on 2 April 2008.

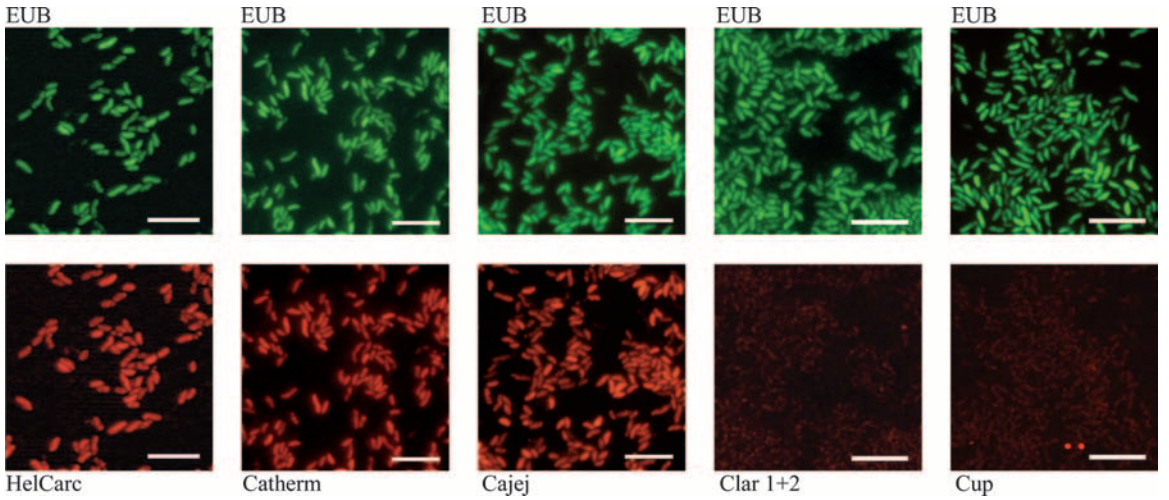

FIG. 1. *C. jejuni* reference strain stained with FISH probes. The results obtained with *C. jejuni* ATCC 33560 are shown. Single slides were each stained simultaneously with the fluorescein isothiocyanate-labeled eubacterial probe (EUB) (green; upper row) and the Cy3-labeled *Campylobacter*-specific probes (red; lower row). Bars, 5 μm.

ously with formamide at a concentration of 30% (10, 21, 23). A FAM-labeled eubacterial probe was always implemented as a control (1). Suspensions of bacteria were prepared in 0.9% saline from overnight cultures on agar plates. Ten microliters of the suspension was applied to glass slides. The slides were air dried and fixed for 20 min in 2% paraformaldehyde. Each slide was hybridized with one specific Cy3-labeled probe in combination with the FAM-labeled eubacterial probe (Fig. 1).

The assay was first evaluated with 12 bacterial reference strains (*C. jejuni* subsp. *jejuni* ATCC 33560, *C. coli* ATCC 33559, *C. lari* ATCC 35221, *C. upsaliensis* ATCC 43954, *C. sputorum* ATCC 35980, *C. concisus* ATCC 33237, *C. fetus* ATCC 27374, *Arcobacter butzleri* ATCC 49616, *Arcobacter cryaerophilus* ATCC 43158, *Arcobacter nitrofigilis* ATCC 33309, *Helicobacter pylori* ATCC 49396, and *Helicobacter pylori* DSMZ 4867). All probes correctly stained the corresponding target reference strains without any cross-reaction with nontarget reference strains.

The probes were further evaluated by using 94 isolates cultured from specimens from humans and animals (Table 2) from the Federal Institute for Risk Assessment, Berlin, Germany; the Animal Sciences Group, Wageningen-Lelystad, The Netherlands; and the Institute of Medical Microbiology, University of Ulm, Ulm, Germany. The isolates were phenotypically characterized by phase-contrast microscopy (characteristic morphology and motility) and Gram staining and by examination of catalase and oxidase production, growth at 25°C and 43°C, indoxyl acetate hydrolysis (22), hippurate hydrolysis (19), and susceptibility to nalidixic acid and cephalothin (5, 14). The identities of three *C. coli* isolates were confirmed by a previously published PCR approach (27).

The corresponding group-specific probe correctly detected all thermotolerant *Campylobacter* isolates without any cross-reaction (Table 2). For the species-specific identification of *C. jejuni*, the assay showed a sensitivity of 90% (26/29 isolates) (Table 2). The *C. jejuni*-specific probe showed one false-positive reaction (1/55 isolates) with a *C. coli* isolate and thus reached a specificity of 98%. The sensitivities of the *C. lari*-specific probe and the *C. upsaliensis*-specific probe were 81% (9/10 isolates) and 100% (11/11 isolates), respectively. The specificity of the *C. lari*- and *C. upsaliensis*-specific probes was 100% (Table 2).

The most striking capacity of the assay was the 100% reliable recognition of thermotolerant *Campylobacter* within less than 2 h with limited effort. From a clinical point of view, the identification of a *Campylobacter* as thermotolerant and, thus, pathogenic is critical in order to initiate adequate therapy and infection control measures. Our results extend a recent report of the successful application of a similar FISH probe for the detection of thermotolerant *Campylobacter* spp. in poultry (25).

We suggest the use of a two-step FISH procedure for the

TABLE 2. Number and percentage of positive FISH results obtained with the isolates tested

Species	No. (%) of the following species (probes):					
	Total	*Helicobacter*, *Campylobacter*, *Arcobacter* (HelCArc)	Thermotolerant *Campylobacter* (Catherm)	*C. jejuni* (Cajej)	*C. upsaliensis* (Cup)	*C. lari* (Clar1 and Clar2)
C. jejuni	29	29 (100)	29 (100)	26 (90)	0 (0)	0 (0)
C. coli	32	32 (100)	32 (100)	1 (3)	0 (0)	0 (0)
C. upsaliensis	10	10 (100)	10 (100)	0 (0)	10 (100)	0 (0)
C. lari	11	11 (100)	11 (100)	0 (0)	0 (0)	9 (81)
Helicobacter pylori	12	12 (100)	0 (0)	0 (0)	0 (0)	0 (0)

TABLE 3. Algorithm for interpretation of FISH results

Result obtained with the following probe (probe specificity):				Interpretation
EUB (all bacteria)	HelCArc (*Helicobacter*, *Campylobacter*, *Arcobacter*)	Catherm (thermotolerant *Campylobacter*)	Cajej (*C. jejuni*)	
+	+	+	+	*C. jejuni*
+	+	+	−	Thermotolerant *Campylobacter* other than *C. jejuni*
+	+	−	−	*Arcobacter*, *Helicobacter*, or nonthermotolerant *Campylobacter*
+	−	−	−	Some bacteria other than *Arcobacter*, *Helicobacter*, or *Campylobacter*
−	−	−	−	No result (the FISH procedure did not work)
+	−	+	−	No result (contradictory binding pattern)

further differentiation of *Campylobacter*. In the first step, the *C. jejuni*-specific probe may be used in combination with the probe specific for thermotolerant *Campylobacter* (the Catherm probe) and with the probe specific for *Campylobacter*, *Arcobacter*, and *Helicobacter* (the HelCArc probe). This step identifies the most frequent isolate, *C. jejuni*, with minimal effort (Table 3). Strains that are negative with the *C. jejuni*-specific probe but positive with all other probes represent thermotolerant *Campylobacter* spp. other than *C. jejuni*. The corresponding strains may be further characterized with considerable reliability in a second step by using the species-specific probes (Table 2). Strains that are negative with the three available species-specific probes may be considered *C. coli*, with a sensitivity of 97% and a specificity of 92% according to the data obtained with our sample collection.

Strains that stain negative with the probe specific for thermotolerant *Campylobacter* spp. (the Catherm probe) but positive with the probe specific for *Campylobacter* and its relatives (the HelCArc probe) represent *Arcobacter*, *Helicobacter*, or nonthermotolerant *Campylobacter* spp. Recognition of these strains provides a considerable advantage, because *Arcobacter* in particular but also nonthermotolerant *Campylobacter* spp. may be confused with thermotolerant *Campylobacter* by biochemical methods (4, 9, 24). The corresponding strains may be further analyzed biochemically or by FISH with previously published probes specific for *Campylobacter* (15, 17), *Arcobacter* (15), and *Helicobacter* (3).

In summary, FISH is suitable for the rapid identification of cultured isolates of thermotolerant *Campylobacter* spp. in a routine laboratory.

This work was supported by a grant from the University of Ulm, Forschungsförderung, to Sven Poppert. This work was partially funded by Seapro Theranostics International B.V., Lelystad, The Netherlands.

We thank Jaap Wagenaar and Jeroen Dijkstra (Animal Sciences Group, Wageningen, Lelystad, The Netherlands) for support and for providing us with *Campylobacter* strains. We thank Damien Lynch, Steffen Stenger, and Len Cegielka for critical reading of the manuscript.

REFERENCES

1. **Amann, R. I., B. J. Binder, R. J. Olson, S. W. Chisholm, R. Devereux, and D. A. Stahl.** 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. **56:**1919–1925.
2. **Barros-Velazquez, J., A. Jimenez, and T. G. Villa.** 1999. Isolation and typing methods for the epidemiologic investigation of thermotolerant campylobacters. Int. Microbiol. **2:**217–226.
3. **Chan, V., G. Crocetti, M. Grehan, L. Zhang, S. Danon, A. Lee, and H. Mitchell.** 2005. Visualization of Helicobacter species within the murine cecal mucosa using specific fluorescence in situ hybridization. Helicobacter **10:** 114–124.
4. **Diergaardt, S. M., S. N. Venter, A. Spreeth, J. Theron, and V. S. Brozel.** 2004. The occurrence of campylobacters in water sources in South Africa. Water Res. **38:**2589–2595.
5. **Fitzgerald, C., and I. Nachamkin.** 2007. *Campylobacter* and *Arcobacter*, p. 933–946. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), Manual of clinical microbiology, 9th ed. ASM Press, Washington, DC.
6. **Gorkiewicz, G., G. Feierl, C. Schober, F. Dieber, J. Kofer, R. Zechner, and E. L. Zechner.** 2003. Species-specific identification of campylobacters by partial 16S rRNA gene sequencing. J. Clin. Microbiol. **41:**2537–2546.
7. **Hill, J. E., A. Paccagnella, K. Law, P. L. Melito, D. L. Woodward, L. Price, A. H. Leung, L. K. Ng, S. M. Hemmingsen, and S. H. Goh.** 2006. Identification of Campylobacter spp. and discrimination from Helicobacter and Arcobacter spp. by direct sequencing of PCR-amplified *cpn60* sequences and comparison to *cpnDB*, a chaperonin reference sequence database. J. Med. Microbiol. **55:**393–399.
8. **Huysmans, M. B., J. D. Turnidge, and J. H. Williams.** 1995. Evaluation of API Campy in comparison with conventional methods for identification of thermophilic campylobacters. J. Clin. Microbiol. **33:**3345–3346.
9. **Jacob, J., I. Feuerpfeil, and E. Schulze.** 1996. PCR-mediated DNA fingerprinting of atypical campylobacter strains isolated from surface and drinking water. Zentralbl. Bakteriol. Parasitenkd. Infektkrankh. Hyg. Abt. 1 Orig. **285:**106–112.
10. **Kempf, V. A., K. Trebesius, and I. B. Autenrieth.** 2000. Fluorescent in situ hybridization allows rapid identification of microorganisms in blood cultures. J. Clin. Microbiol. **38:**830–838.
11. **Kuijper, E. J., S. Stevens, T. Imamura, B. De Wever, and E. C. Claas.** 2003. Genotypic identification of erythromycin-resistant *Campylobacter* isolates as *Helicobacter* species and analysis of resistance mechanism. J. Clin. Microbiol. **41:**3732–3736.
12. **Kulkarni, S. P., S. Lever, J. M. Logan, A. J. Lawson, J. Stanley, and M. S. Shafi.** 2002. Detection of Campylobacter species: a comparison of culture and polymerase chain reaction based methods. J. Clin. Pathol. **55:**749–753.
13. **Logan, J. M., K. J. Edwards, N. A. Saunders, and J. Stanley.** 2001. Rapid identification of *Campylobacter* spp. by melting peak analysis of biprobes in real-time PCR. J. Clin. Microbiol. **39:**2227–2232.
14. **Luber, P., J. Wagner, H. Hahn, and E. Bartelt.** 2003. Antimicrobial resistance in *Campylobacter jejuni* and *Campylobacter coli* strains isolated in 1991 and 2001–2002 from poultry and humans in Berlin, Germany. Antimicrob. Agents Chemother. **47:**3825–3830.
15. **Moreno, Y., S. Botella, J. L. Alonso, M. A. Ferrus, M. Hernandez, and J. Hernandez.** 2003. Specific detection of *Arcobacter* and *Campylobacter* strains in water and sewage by PCR and fluorescent in situ hybridization. Appl. Environ. Microbiol. **69:**1181–1186.
16. **Moreno, Y., M. A. Ferrus, J. L. Alonso, A. Jimenez, and J. Hernandez.** 2003. Use of fluorescent in situ hybridization to evidence the presence of Helicobacter pylori in water. Water Res. **37:**2251–2256.
17. **Moreno, Y., M. Hernandez, M. A. Ferrus, J. L. Alonso, S. Botella, R. Montes, and J. Hernandez.** 2001. Direct detection of thermotolerant campylobacters in chicken products by PCR and in situ hybridization. Res. Microbiol. **152:** 577–582.
18. **Moter, A., and U. B. Gobel.** 2000. Fluorescence in situ hybridization (FISH) for direct visualization of microorganisms. J. Microbiol. Methods **41:**85–112.
19. **Nicholson, M. A., and C. M. Patton.** 1995. Evaluation of disk method for hippurate hydrolysis by *Campylobacter* species. J. Clin. Microbiol. **33:**1341–1343.
20. **On, S. L., and P. J. Jordan.** 2003. Evaluation of 11 PCR assays for species-level identification of *Campylobacter jejuni* and *Campylobacter coli*. J. Clin. Microbiol. **41:**330–336.

21. **Pernthaler, J., F. O. Gloeckner, W. Schoenhuber, and R. Amann.** 2006. Fluorescence in situ hybridization with rRNA-targeted oligonucleotide probes. Fluorescence in situ hybridization. Methods Microbiol. Mar. Microbiol. **30:**1–31.
22. **Popovic-Uroic, T., C. M. Patton, M. A. Nicholson, and J. A. Kiehlbauch.** 1990. Evaluation of the indoxyl acetate hydrolysis test for rapid differentiation of *Campylobacter*, *Helicobacter*, and *Wolinella* species. J. Clin. Microbiol. **28:**2335–2339.
23. **Poppert, S., A. Essig, B. Stoehr, A. Steingruber, B. Wirths, S. Juretschko, U. Reischl, and N. Wellinghausen.** 2005. Rapid diagnosis of bacterial meningitis by real-time PCR and fluorescence in situ hybridization. J. Clin. Microbiol. **43:**3390–3397.
24. **Prouzet-Mauleon, V., L. Labadi, N. Bouges, A. Menard, and F. Megraud.** 2006. Arcobacter butzleri: underestimated enteropathogen. Emerg. Infect. Dis. **12:**307–309.
25. **Schmid, M. W., A. Lehner, R. Stephan, K. H. Schleifer, and H. Meier.** 2005. Development and application of oligonucleotide probes for in situ detection of thermotolerant Campylobacter in chicken faecal and liver samples. Int. J. Food Microbiol. **105:**245–255.
26. **Snaidr, J., R. Amann, I. Huber, W. Ludwig, and K. H. Schleifer.** 1997. Phylogenetic analysis and in situ identification of bacteria in activated sludge. Appl. Environ. Microbiol. **63:**2884–2896.
27. **Wang, G., C. G. Clark, T. M. Taylor, C. Pucknell, C. Barton, L. Price, D. L. Woodward, and F. G. Rodgers.** 2002. Colony multiplex PCR assay for identification and differentiation of *Campylobacter jejuni*, *C. coli*, *C. lari*, *C. upsaliensis*, and *C. fetus* subsp. *fetus*. J. Clin. Microbiol. **40:**4744–4747.