APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 2003, p. 5664–5669 Vol. 69, No. 9
0099-2240/03/$08.00+0 DOI: 10.1128/AEM.69.9.5664–5669.2003

Toward an International Standard for PCR-Based Detection of Food-Borne Thermotolerant Campylobacters: Assay Development and Analytical Validation

P. S. Lübeck,[1] P. Wolffs,[2] S. L. W. On,[1] P. Ahrens,[1] P. Rådström,[2] and J. Hoorfar[1]*

Danish Veterinary Institute, DK-1790 Copenhagen, Denmark,[1] and Applied Microbiology, Lund University, 22100 Lund, Sweden[2]

Received 29 January 2003/Accepted 23 June 2003

As part of a European research project (FOOD-PCR), we developed a standardized and robust PCR detection assay specific for the three most frequently reported food-borne pathogenic *Campylobacter* species, *C. jejuni*, *C. coli*, and *C. lari*. Fifteen published and unpublished PCR primers targeting the 16S rRNA gene were tested in all possible pairwise combinations, as well as two published primers targeting the 23S rRNA gene. A panel of 150 strains including target and nontarget strains was used in an in-house validation. Only one primer pair, OT1559 plus 18-1, was found to be selective. The inclusivity and exclusivity were 100 and 97%, respectively. In an attempt to find a thermostable DNA polymerase more resistant than *Taq* to PCR inhibitors present in chicken samples, three DNA polymerases were evaluated. The DNA polymerase T*th* was not inhibited at a concentration of 2% (vol/vol) chicken carcass rinse, unlike both *Taq* DNA polymerase and DyNAzyme. Based on these results, T*th* was selected as the most suitable enzyme for the assay. The standardized PCR test described shows potential for use in large-scale screening programs for food-borne *Campylobacter* species under the assay conditions specified.

The species *Campylobacter jejuni*, *C. coli*, and *C. lari* are among the most frequently reported food-borne pathogens in humans worldwide (7). They can be found in a wide range of foods, including poultry, pig, beef, and seafood products, with chicken meat considered the most common source of human infection (12, 22). Effective methods for detecting these bacteria in foods are important tools for protecting the public health; however, detection of *Campylobacter* by standard isolation methods is problematic. In samples such as food, the agent may be present in low numbers, and the organisms are relatively sensitive to environmental factors, such as atmospheric oxygen, low pH, dryness, and temperature (22). Consequently, the number of viable *Campylobacter* cells can be rapidly and substantially reduced during storage or transportation of food samples to testing laboratories (18). Moreover, antibiotics used to improve the selectivity of culture media may inhibit the growth of certain strains if they are sensitive to one or more of the selective agents (8).

The application of culture-independent detection methods such as PCR may help to overcome the aforementioned problems (15). In addition, PCR in general provides faster results than conventional culture and has the potential for automation (9, 27). The latter is necessary for application of the test in large-scale screening programs in which many samples are examined in a short period of time. Many diagnostic laboratories have developed PCR-based methods for pathogen detection (5, 6, 9, 23, 28, 29), but many variables may affect the efficacy of PCR, and the results of tests developed or published by one laboratory can sometimes be difficult to reproduce by other laboratories (21). Moreover, PCR inhibitors originating from food samples may be difficult to overcome in PCR protocols using conventional enzymes: e.g., *Taq* polymerases (1). This may include testing different DNA polymerases in the matrices chosen for the study with the aim of identifying a polymerase that best overcomes the present inhibitors and validation of an internal amplification control (IAC) to identify false-negative responses. Proper validation based on consensus criteria is therefore an absolute prerequisite for successful adoption of PCR-based diagnostic methodology (10).

One of the aims of the European FOOD-PCR project (www.pcr.dk) was to evaluate and validate noncommercial PCR assays for the specific detection of *C. jejuni*, *C. coli*, and *C. lari* in foods. The present study evaluated 17 published and unpublished PCR primers targeting various rRNA gene regions. An extensive in-house validation was carried out through a new combination of published primers. Furthermore, in order to find a suitable enzyme resistant to inhibition by chicken samples, three DNA polymerases were investigated.

MATERIALS AND METHODS

Terms. The terms used in this study refer to conventions described in the MicroVal protocol (3). Selectivity was defined as a measure of the degree of response from target and nontarget microorganisms and comprises inclusivity and exclusivity. Inclusivity describes the ability of a method (here PCR) to specifically detect the target pathogen from a wide range of strains, whereas exclusivity is the lack of response from a relevant range of closely related, nontarget strains (10). According to the new International Organization for Standardization (ISO) standard (3, 17), the terms "inclusivity" and "exclusivity" should replace the traditional terms "specificity" and "sensitivity," which should only be used to express results from diagnostic samples (10).

Bacterial strains, growth conditions, and DNA extraction. One hundred fifty strains (mainly *Campylobacter* spp.) were used in this study (Table 1). These included type, reference, and well-characterized field strains from various sources, including chickens, pigs, and cattle in Denmark, identified by conventional and molecular methods (19). All *Campylobacter* strains were cultured on

* Corresponding author. Mailing address: Danish Veterinary Institute, Bülowsvej 27, DK-1790 Copenhagen V, Denmark. Phone: 45-35 300 251. Fax: 45-35 300 120. E-mail: jho@vetinst.dk.

TABLE 1. List of strains used for the development and validation of the PCR used in this study[a]

No.	Species	Sero/biotype	Strain	No.	Species	Sero/biotype	Strain
1*	*C. jejuni*	Penner 1	CCUG 10935	76**	*C. lari*	NARTC[c]	CCUG 15035
2*	*C. jejuni*	Penner 2	CCUG 10936	77**	*C. lari*	NARTC	CCUG 12774
3*	*C. jejuni*	Penner 3	CCUG 10937	78**	*C. lari*	NARTC	CCUG 23949
4*	*C. jejuni*	Penner 4	CCUG 10938	79*	*C. lari*	NARTC	CCUG 19528
5*	*C. jejuni*	Penner 5	CCUG 10959	80*	*C. lari*	NARTC	CCUG 23948
6*	*C. coli*	Penner 5	CCUG 10939	81*	*C. lari*	NARTC	SVS 98-40052
7*	*C. jejuni*	Penner 6	CCUG 12778	82*	*C. lari*	UPTC[d]	CCUG 20707
8*	*C. jejuni*	Penner 7	CCUG 10940	83*	*C. lari*	UPTC	CCUG 18267
9*	*C. jejuni*	Penner 8	CCUG 16436	84*	*C. lari*	UPTC	CCUG 22395
10*	*C. jejuni*	Penner 9	CCUG 10942	85*	*C. lari*	UPTC	CCUG 18294
11*	*C. jejuni*	Penner 10	CCUG 10943	86*	*C. lari*	UPTC	CCUG 22396
12*	*C. jejuni*	Penner 11	CCUG 10944	87*	*C. lari*	UPTC	LU 6/3BW1
13*	*C. jejuni*	Penner 12	CCUG 17625	88*	*C. lari*	UPTC	LU 21/12OC3
14*	*C. jejuni*	Penner 13	CCUG 10945	89*	*C. lari*	UPTC	LU 16/1BTG4
15*	*C. coli*	Penner 14	CCUG 15360	90*	*C. lari*	UPTC	LU 18/3BTG8
16*	*C. jejuni*	Penner 15	CCUG 10946	91*	*C. lari*	UPTC	LU 21/12LW18
17*	*C. jejuni*	Penner 16	CCUG 10947	92*	*C. lari*	UPTC	LU 16/1OC3
18*	*C. jejuni*	Penner 17	CCUG 10948	93	*C. jejuni*	2	7231127-3
19*	*C. jejuni*	Penner 18	CCUG 10949	94	*C. jejuni*	4 complex	7231127-2
20*	*C. jejuni*	Penner 19	CCUG 10950	95	*C. coli*	46	9831503-2
21*	*C. coli*	Penner 20	CCUG 10951	96	*C. coli*	59	9831091-1
22*	*C. jejuni*	Penner 21	CCUG 10952	97	*C. hyointestinalis*		9930731-1
23*	*C. jejuni*	Penner 22	CCUG 10953	98	*C. jejuni*	35	7230701-6
24*	*C. jejuni*	Penner 23	CCUG 10954	99	*C. jejuni*	23,36	7230127-3
25*	*C. coli*	Penner 24	CCUG 10955	100	*C. coli*	51	7231059-1
26*	*C. coli*	Penner 25	CCUG 10956	101	*C. coli*	5	7231058-1
27*	*C. coli*	Penner 26	CCUG 10957	102	*C. hyointestinalis*		9930111-1
28*	*C. jejuni*	Penner 27	CCUG 10958	103	*C. jejuni*	11	99042253-4
29*	*C. coli*	Penner 28	CCUG 10959	104	*C. jejuni*	31	9904313-14
30*	*C. jejuni*	Penner 29	CCUG 15361	105	*C. coli*	5	9904602-19
31*	*C. coli*	Penner 30	CCUG 10960	106	*C. coli*	46	9904253-1
32*	*C. jejuni*	Penner 31	CCUG 10961	107	*C. jejuni*	29	7231125-2
33*	*C. jejuni*	Penner 32	CCUG 10962	108	*C. jejuni*	5	7231120-5
34*	*C. jejuni*	Penner 33	CCUG 10963	109	*C. jejuni*	19	723723-2
35*	*C. coli*	Penner 34	CCUG 10964	110	*C. jejuni*	4 complex	9904253-5
36*	*C. jejuni*	Penner 35	CCUG 10965	111	*C. jejuni*	6,7	9930116-6
37*	*C. jejuni*	Penner 36	CCUG 10966	112	*C. coli*	30	7230141-6
38*	*C. jejuni*	Penner 37	CCUG 10967	113	*C. coli*	46	7231033-1
39*	*C. jejuni*	Penner 38	CCUG 10968	114	*C. coli*	NT	9631023-2
40*	*C. coli*	Penner 39	CCUG 10969	115	*C. coli*	59	7231135-2
41*	*C. jejuni*	Penner 40	CCUG 10970	116	*C. coli*	48	9631038-4
42*	*C. jejuni*	Penner 41	CCUG 10971	117	*C. hyointestinalis*		7230324-3
43*	*C. jejuni*	Penner 42	CCUG 12782	118	*C. hyointestinalis*		9731034-3
44*	*C. jejuni*	Penner 43	CCUG 12783	119	*H. pullorum*-like		9831306-5
45*	*C. jejuni*	Penner 44	CCUG 14567	120	*C. lari*		9831299-3
46*	*C. jejuni*	Penner 45	CCUG 17753	121	*H. pullorum*-like		9831276-8
47*	*C. coli*	Penner 46	CCUG 15362	122**	*C. upsaliensis*	Type strain	CCUG 14913
48*	*C. coli*	Penner 47	CCUG 17715	123**	*C. upsaliensis*		CCUG 33890
49*	*C. coli*	Penner 48	CCUG 17754	124**	*C. upsaliensis*		CCUG 20818
50*	*C. coli*	Penner 49	CCUG 17755	125**	*C. upsaliensis*		CCUG 23017
51*	*C. jejuni*	Penner 50	CCUG 12790	126**	*C. upsaliensis*		CCUG 19559
52*	*C. coli*	Penner 51	CCUG 12791	127**	*C. helveticus*	Type strain	CCUG 30682
53*	*C. jejuni*	Penner 52	CCUG 12792	128**	*C. helveticus*		CCUG 34016
54*	*C. jejuni*	Penner 53	CCUG 15013	129**	*C. helveticus*		CCUG 30563
55*	*C. coli*	Penner 54	CCUG 12794	130**	*C. helveticus*		CCUG 30564
56*	*C. jejuni*	Penner 55	CCUG 12795	131**	*C. helveticus*		CCUG 30565
57*	*C. coli*	Penner 56	CCUG 14537	132**	*C. helveticus*		CCUG 30566
58*	*C. jejuni*	Penner 57	CCUG 14538	133**	*C. helveticus*		CCUG 30683
59*	*C. jejuni*	Penner 58	CCUG 14539	134**	*C. helveticus*		CCUG 34042
60*	*C. coli*	Penner 59	CCUG 14540	135	*C. hyointestinalis*	Type strain	CCUG 14169
61*	*C. jejuni*	Penner 60	CCUG 14541	136	*C. lanienae*	Type strain	NCTC 13004
62*	*C. coli*	Penner 61	CCUG 24865	137	*C. mucosalis*	Type strain	CCUG 6822
63*	*C. jejuni*	Penner 62	CCUG 24866	138	*C. fetus*	Type strain	CCUG 6823
64*	*C. jejuni*	Penner 63	CCUG 24867	139	*C. concisus*	Type strain	CCUG 13144
65*	*C. jejuni*	Penner 64	CCUG 24868	140	*C. curvus*	Type strain	CCUG 13146
66*	*C. jejuni*	Penner 65	CCUG 24869	141	*C. showae*	Type strain	CCUG 30254
67**	*C. jejuni*	Type strain	CCUG 11284	142	*C. rectus*	Type strain	CCUG 20446
68*	*C. jejuni*	C. jejuni[b]	CCUG 24567	143	*C. gracilis*	Type strain	CCUG 27720
69*	*C. jejuni*	C. jejuni[b]	CCUG 18265	144	*Arcobacter butzleri*	Type strain	CCUG 30483
70*	*C. jejuni*	C. jejuni[b]	CCUG 18266	145	*Helicobacter pylori*		Rigsh. 15893
71*	*C. jejuni*	C. jejuni[b]	CCUG 26155	146	*H. pullorum*	Type strain	CCUG 33837
72*	*C. jejuni*	C. jejuni[b]	CCUG 26152	147	*Escherichia coli*		JEO 908149
73*	*C. jejuni*	C. jejuni[b]	SSI 5384	148	*L. monocytogenes*		JEO 2268-179
74**	*C. coli*	Type strain	CCUG 11283	149	*Y. enterocolitica*		JH2 O:3
75*	*C. lari*	Type strain	CCUG 23947	150	*S. enterica*		CCUG 31969

[a] *, strains used for testing the four published PCR methods; **, strains used in the preliminary study for identifying the primer pair with the best selectivity.
[b] *C. jejuni*, subsp. *doylei* strain.
[c] NARTC, nalidixic acid-resistant thermophilic *Campylobacter*.
[d] UPTC, urease-positive thermophilic *Campylobacter*.

TABLE 2. Primers used in different combinations to develop the best PCR assay for detection of *C. jejuni*, *C. coli*, and *C. lari*

Target	Primer (sequence)	Reference
23S	Therm1 (5′-TATTCCAATACCAACATTAGT) Therm4 (5′-CTTCGCTAATGCTAACCC)	6
16S	6–1 (5′-GTCGAACGATGAAGCTTCTA) 18–1 (5′-TTCCTTAGGTACCGTCAGAA)	5
16S	CF1 (5′-GGAAGGATGACACTTTTCGGA GCG) CR2 (5′-TCGCGGTATTGCGTCTCATTGTA TATGC)	28
16S	C442 (5′-GGAGGATGACACTTTTCGG AGC) C490 (5′-ATTACTGAGATGACTAGCAC CCC)	29
16S	OT1559 (5′-CTGCTTAACACAAGTTGAGT AGG)	25
16S	18-1rev (5′-TTCTGACGGTACCTAAGGAA)	This study
16S	CF1rev (5′-CGCTCCGAAAAGTGTCATCC TCC)	This study
16S	C490rev (5′-GGGGTGGCTAGCCATCTCAG TATT)	This study
16S	JCL-1 (5′-ATAGTTAATCTGCCCTACACAA)	This study
16S	JCL-2 (5′-TCCTTTTCTTAGGGAAGAATTC)	This study
16S	JCL-3 (5′-CGTCAGAATTCTTCCCTAAG)	This study
16S	JCL-4 (5′-AGTTTAGTATTCCGGCTTCGA)	This study
16S	JCL-5 (5′-GATTCCACTGTGGACGGTAA)	This study
IAC[a]	CCL-f (5′-OT1559-GGTTCATGAGGACACC TGAGTT)	This study
	CCR-r (5′-18-1-TATACACTCTCATCCCTCC AAC)	This study

[a] IAC, primers used for construction of the internal amplification control.

5% calf blood agar plates (CM331; Oxoid, Basingstoke, United Kingdom) under microaerobic conditions (6% O_2, 7% CO_2, 7% H_2, 80% N_2). All non-*Campylobacter* strains were grown in Luria-Bertani (LB) medium prepared from 5 g of sodium chloride, 5 g of yeast extract (L21; Oxoid) and 10 g of tryptone peptone (211705; Difco, Detroit, Mich.) dissolved in 1,000 ml of distilled water. The pH was adjusted to 7.3 to 7.4. The strains were stored as frozen cell suspensions in LB medium-glycerol (1:1) at −80°C. DNA was extracted from 2- to 3-day-old bacterial growth by using protocol no. 3 of the Easy-DNA kit (K1800-01; Invitrogen, Carlsbad, Calif.).

Selection of published primers. rRNA gene sequences of *C. jejuni*, *C. coli*, and *C. lari* consistently share extensive homology, but are more distinct from other *Campylobacter* spp. (20). Thus, one probe (25) and three primer sets (Table 2) targeting 16S and one primer pair targeting 23S ribosomal DNA (rDNA) (4, 5, 24, 25) were tested on 105 *Campylobacter* isolates (Table 1). For testing the published PCR assays, the reaction conditions used, including temperature profile and DNA polymerase, were essentially as described in the original publications. The thermocycler used in this and subsequent studies was a GeneAmp PCR system 9700 (Applied Biosystems, Weiterstadt, Germany). After cycling, the PCR amplicons in this and subsequent studies were detected by electrophoresis in 1.8% agarose gels stained with ethidium bromide.

New primer combinations. Since none of the published primer sets resulted in the required selectivity (see Results), new primer combinations were tested with 18 strains (Table 1), chosen to identify assays capable of detecting *C. jejuni*, *C. coli*, and *C. lari*, but not the closely related, but not food-borne species *C. upsaliensis* and *C. helveticus.* The following PCR mixture (50 μl) was used: 10× PCR buffer, 0.2 mM deoxynucleoside triphosphates (dNTPs) (27-2035-03; Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom), 0.2 μM each primer, 0.4 U of DNA polymerase, and 3 mM $MgCl_2$ (N808-0010; Applied Biosystems, Nærum, Denmark), and 1.0 μl of target DNA solution. The thermocycling program comprised an initial denaturation (94°C, 2 min) followed by 35 cycles of denaturation at (94°C, 1 min), annealing (55°C, 1 min), and extension at (72°C, 1 min). A final extension cycle (72°C, 4 min) completed the PCR. For preliminary optimization of the PCR and cycling parameters, the type strain of *C. jejuni* (CCUG 11284) was used. The optimized PCR mixture in 25 μl contained 10× PCR buffer for T*th* DNA polymerase (1480022; Roche Applied Science, Hvidovre, Denmark), 0.2 mM dNTP, 0.22 μM primer OT1559, 0.24 μM primer 18-1, 1 U of T*th* DNA polymerase (14800322; Roche Applied Science), 5 μg of bovine serum albumin (20 mg/ml) (711454; Roche Applied Science), 2 mM $MgCl_2$, 0.25 μl (≅10^3 copies) of an internal control DNA (described below), and 1 μl of target sample DNA solution (≅100 pg ≅ 5×10^4 copies). All PCRs were made in triplicate in 0.2-ml PCR tubes.

Final standard PCR. The most selective new combination of primers OT1559 and 18-1 (see Results) was chosen as the final standard test. The final thermocycling program was as follows: initial denaturation 94°C at 2 min; then 35 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 15 s, and extension at 72°C for 30 s; and finally an extension at 72°C for 4 min.

Sequencing. The 16S rRNA gene sequence from strain CCUG 19559 was determined by a sequencing method as described previously (2). Alignment and numerical comparison of this sequence with GenBank database sequences of the type strains of all 16 *Campylobacter* species were performed with the program BioNumerics v2.5 (Applied Maths, Kortrijk, Belgium) using both default parameters and those described previously (20).

Construction of internal amplification control. A 124-bp internal amplification control (IAC) amplicon was constructed based on DNA from the viral hemorrhagic septicema virus (GenBank accession no. X66134). This DNA was chosen since it is not found in food samples and has shown to work well previously (11). The IAC was produced in 50-μl reaction mixtures comprising 10 mM Tris (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM dNTP, 0.1 μM each primer, 0.5 U of *Taq* polymerase (1146165; Roche Applied Science), and 2 μl of target DNA sample. The thermocycling program was as follows: 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. The PCR products were purified from the agarose gel by using the QIAquick gel extraction kit (28704; Qiagen, Hilden, Germany) and finally eluated in 50 μl of sterile water. The fragment was furthermore cloned in plasmid pCR2.1 by using the TA cloning kit and One Shot TOP10 competent cells as recommended by the supplier (K2040-01; Invitrogen, Carlsbad, Calif.). Plasmids were recovered with the QIAprep Spin Miniprep kit (27104; Qiagen).

DNA polymerase and PCR inhibition test. To identify a DNA polymerase resistant to PCR inhibitors present in chicken samples, a previously standardized PCR assay specific for pathogenic *Yersinia enterocolitica* was used (14). Different concentrations (from 1 fg/ml to 1 mg/ml) of DNA isolated from *Y. enterocolitica* Y79 (14) were added to the amplification mixture containing different percentage dilutions (vol/vol) of the chicken rinse sample (Table 3). All chicken samples had been certified free from naturally occurring pathogenic *Y. enterocolitica* by PCR. Whole chicken carcasses or neck skins were obtained from slaughterhouses or retailers in Denmark or Sweden. The chicken rinse samples comprised whole chickens washed in 500 ml of buffered peptone water (BPW) or sterile saline as described previously (13). Chicken neck skin samples were prepared by adding 10 g of neck skin to 100 ml of BPW or saline, homogenizing it in a stomacher for 30 s, and removing the skin sample. To test the PCR inhibitory effect of these samples, aliquots were added to the PCR mixture in a final concentration of 20% (vol/vol). Also, the inhibition of 10- and 100-fold-diluted chicken carcass rinse samples (respectively, 2 and 0.2% in the PCR mixture) were tested. Two potentially resistant enzymes, along with *Taq*, were tested (1). DyNAzyme (F501L; Finnzymes, Espoo, Finland), *Taq* and T*th* DNA polymerases and accompanying buffer systems were evaluated for resistance to the inhibitory effect of chicken carcass rinse matrix. For real-time PCR, a LightCycler instrument (Roche Diagnostics) and a real-time assay based on the same *Y. enterocolitica* primer pair were used. The PCR mix contained 10× buffer supplied with the appropriate DNA polymerase (*Taq*, T*th*, or DyNAzyme), 2.5 U of enzyme, 4 mM $MgCl_2$, 0.44 μM each primer, 0.2 mM each dNTP, 10,000-times-diluted SYBR Green I (1988131; Roche Applied Science), and 4 μl of sample. The total volume was 20 μl. The amplification conditions included a denaturation step of 1 min at 95°C, followed by 40 cycles of 0.1 s of denaturation at 95°C, 5 s of annealing at 60°C, and 15 s of elongation at 72°C, followed by a single fluorescent measurement and finally 25 s of final elongation. Amplification was followed by a melting curve

TABLE 3. Effect of inhibition by carcass rinse from chicken on PCR amplification with different DNA polymerases in a *Y. enterocolitica* PCR assay

DNA polymerase	Rinse sample (%)[a]	Result with *Y. enterocolitica* concen (CFU/25-μl reaction tube)[d]							
		Conventional PCR[b]				Real-time PCR[c]			
		5×10^3	5×10^2	5×10^1	5×10^0	5×10^3	5×10^2	5×10^1	5×10^0
Taq polymerase	Water	++	++	++	+−	++	++	++	−−
	20	−−	−−	−−	−−	−−	−−	−−	−−
	2	+−	−−	−−	−−	++	−−	−−	−−
	0.2	+−	++	+−	−−	++	++	++	−−
T*th* polymerase	20	−−	−−	−−	−−	−−	−−	−−	−−
	2	++	++	++	−−	++	++	++	−−
	0.2	++	++	++	−−	++	++	++	−−
DyNAzyme II	20	−−	−−	−−	−−	−−	−−	−−	−−
	2	−−	++	−−	−−	++	++	−−	−−
	0.2	++	++	++	+−	++	++	++	−−

[a] Carcass-rinse sample from neck skin of chicken in physiological saline.
[b] Two independent PCR results confirmed by gel electrophoresis.
[c] Two independent PCR results confirmed by melting-curve analysis in a LightCycler instrument.
[d] Overnight culture of *Y. enterocolitica* Y79 in Tris-buffered saline with CFU determination by plating on TGE plates. Dilutions of the cell suspensions were made in physiological saline and added to the PCR mixture to a final amount of 20% (vol/vol).

analysis between 65 and 95°C and finally a cooling step for 1 min at 40°C. During amplification, the fluorescence was measured by using gain setting F1:1 with display mode F1.

RESULTS

Selectivity. The results of the inclusivity and exclusivity tests are presented in Table 4. Only one primer pair, OT1559 plus 18-1, showed adequate selectivity. This primer pair was then tested in PCR against all 150 strains to verify its selectivity. The results showed that the inclusivity was 100%, whereas the exclusivity was 97%; only *C. upsaliensis* strain CCUG 19559 resulted in a positive PCR amplification. A comparison of the 16S rDNA sequence of strain CCUG 19559 and those of *C. upsaliensis*, *C. jejuni*, *C. coli*, and *C. lari* showed that the two

TABLE 4. Results from the preliminary screening of 26 primer combinations against DNA from 18 isolates and strains

Primer pair	Screening results for:						
	Inclusivity (no. of strains)				Exclusivity (no. of strains)		
	C. jejuni ($n = 1$)	*C. coli* ($n = 1$)	*C. lari* ($n = 3$)	No. of positive target strains/total true positive (%)	*C. upsaliensis* ($n = 5$)	*C. helveticus* ($n = 8$)	No. of negative nontarget strains/total true negative (%)
6-1 + 18-1	1	1	3	5/5 (100)	5	8	0/13 (0)
CF1 + CR2	1	0	3	4/5 (80)	4	8	1/13 (8)
C442 + C490	1	0	3	4/5 (80)	5	8	0/13 (0)
18-1rev + CR2	1	0	3	4/5 (80)	5	8	0/13 (0)
18-1rev + C490	1	0	3	4/5 (80)	5	8	0/13 (0)
OT1559 + 18-1[a]	1	1	3	5/5 (100)	1	0	12/13 (92)
OT1559 + C490	1	0	3	4/5 (80)	1	1	11/13 (85)
OT1559 + CR2	1	0	3	4/5 (80)	1	1	11/13 (85)
6-1 + CR2	1	0	3	4/5 (80)	5	8	0/13 (0)
JCL-1 + JCL-3	1	1	3	5/5 (100)	2	6	5/13 (39)
JCL-1 + JCL-4	0	0	0	0/5 (0)	0	0	13/13 (100)
JCL-1 + JCL-5	0	0	0	0/5 (0)	0	0	13/13 (100)
JCL-2 + JCL-4	1	0	2	3/5 (60)	0	1	12/13 (92)
JCL-2 + JCL-5	0	0	0	0/5 (0)	0	1	12/13 (92)
OT1559 + JCL3	1	1	3	5/5 (100)	2	5	6/13 (46)
OT1559 + JCL4	0	0	0	0/5 (0)	0	0	13/13 (100)
OT1559 + JCL5	0	0	0	0/5 (0)	0	1	12/13 (92)
JCL-1 + 18-1	1	1	3	5/5 (100)	2	6	5/13 (39)
C490rev + JCL-4	1	0	3	4/5 (80)	4	7	2/13 (15)
C490rev + JCL-5	1	0	3	4/5 (80)	2	2	9/13 (69)
18-1rev + JCL-4	0	0	0	0/5 (0)	1	1	11/13 (85)
18-1rev + JCL-5	0	0	1	1/5 (20)	1	2	10/13 (77)
6-1 + CF1rev	1	0	0	1/5 (20)	1	8	4/13 (31)
JCL-1 + CF1rev	1	1	3	5/5 (100)	4	8	1/13 (8)
OT1559 + CF1rev	1	1	3	5/5 (100)	2	4	7/13 (54)
C490 + CR2	0	0	0	0/5 (0)	0	0	13/13 (100)

[a] Note that the OT1559 + 18-1 primer combination was the most selective new combination and was chosen as the final standard test.

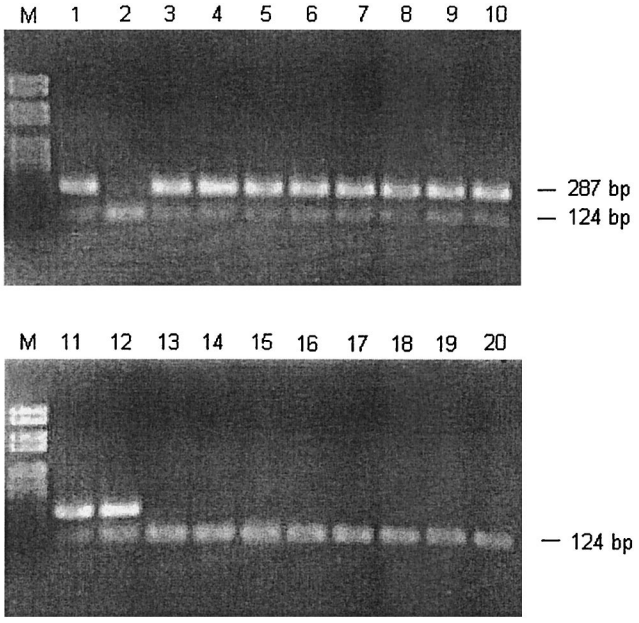

FIG. 1. Result of the optimized PCR protocol detecting *C. jejuni*, *C. coli*, and *C. lari* (upper band), including the internal control (lower band). Lanes: M, molecular size marker; 1, positive control (CCUG 11284); 2, negative control (only internal control is added); 3 to 12, different strains of *C. jejuni*, *C. coli*, and *C. lari*; 13 to 20, other *Campylobacter* and non-*Campylobacter* species (strains marked with double asterisks in Table 1).

primer annealing sites in strain CCUG 19559 were identical to sequences of the latter group of species. However, the 16S rRNA sequences of CCUG 19559 differed by 6 and 2 bp, respectively, in the primer-binding region compared to seven published *C. upsaliensis* 16S rRNA sequences. Nonetheless, the ca. 1,500-bp segment of the CCUG 19559 16S rDNA gene sequence was found to be 98.4% similar to *C. upsaliensis* 16S rDNA, compared with a corresponding value of 96.5% similarity for other *C. jejuni* strains.

IAC and detection limit. A dilution series of the purified IAC fragment was made to determine the detection level in the final PCR. The IAC was coamplified with the target DNA (*C. jejuni* CCUG 11284) at 287 bp (Fig. 1). The detection limits for the IAC were 2.2×10^{-17} g (50 to 100 copies) when it was amplified alone and 2.2×10^{15} g (5×10^3 copies) when it was amplified together with 10 pg of target DNA by using 35 amplification cycles. The detection limit for the target DNA (*C. jejuni* CCUG 11284) was 3.1×10^{-14} g (17 copies, assuming a genome size of 1.64×10^6 bp) when it was amplified without IAC.

Evaluation of thermostable DNA polymerases. Undiluted chicken rinse 20% (vol/vol) was found to completely inhibit PCR independent of the concentration of the target or the DNA polymerase was used. *Taq* and DyNAzyme polymerases were not inhibited when the chicken rinse was added to the PCR at a concentration of 0.2% (vol/vol), while T*th* DNA polymerase showed no inhibition at a concentration of 2% (vol/vol) chicken rinse. Similar results were obtained for conventional and real-time PCR. Based on these results, T*th* was selected as the most suitable DNA polymerase for the final *Campylobacter* PCR assay.

DISCUSSION

Selectivity was the principal criterion used to identify a PCR test for international validation as a tool for rapid and effective detection of *C. jejuni*, *C. coli*, and *C. lari* in foods. We aimed to identify an assay that included all strains of these three predominant food-borne *Campylobacter* species, but excluded all other species. The four published PCR assays evaluated based upon 16S and 23S rRNA gene sequences lacked the accepted selectivity to food-borne campylobacters, since they also yielded amplicons for *C. upsaliensis* and *C. helveticus.* This was considered disadvantageous, since domestic pets are the only known animal reservoir for these taxa, and *C. helveticus* has not yet been reported from human disease (19). Similar results have been reported with 23S rRNA gene sequence-derived PCR tests first proposed as selective for the identification of *C. jejuni* and *C. coli* in a study that emphasized the need for appropriate strain selection in the validation process (21). Subsequently, new combinations of existing primers, together with new primers were tested to improve the selectivity for *C. jejuni*, *C. coli*, and *C. lari* (Table 1). The primer pair with a 100% inclusivity score and the best exclusivity score was then tested in PCR against all 150 strains (Table 1) to assess its overall selectivity (Table 4). However, it was observed that two strains of *C. helveticus* appeared with a faint, nearly invisible band when an annealing temperature of 55°C was used, but these amplicons were not obtained with the final optimized cycling parameters (30 s at 94°C, 15 s at 58°C, and 30 s at 72°C for 35 cycles). After the change to these conditions, only one *C. upsaliensis* strain (CCUG 19559) was still detected by the assay. Given that both *C. upsaliensis* and *C. helveticus* are highly related to the food-borne species *C. jejuni*, *C. coli*, and *C. lari* (19, 20), this result is not altogether unexpected. A recent publication (4) showed that 28 of 29 *hipO*-negative *Campylobacter* isolates possessed 16S rRNA genes that were indistinguishable from those of *C. jejuni* type strains (based on 16S rRNA restriction fragment length polymorphism data). These *hipO*-negative isolates were found to be *C. coli* by the cumulative evidence of six published PCR-based assays, suggesting that speciation data based solely on this gene should be interpreted with caution. Furthermore, four 16S rRNA genes from *hipO*-negative strains were sequenced, which showed that they were almost identical to *C. jejuni* type strain 16S rRNA sequences deposited in GenBank. This observation is important given that others have reported problems with phylogenetic analyses of bacterial species based solely on 16S rRNA gene sequence comparison (26).

Moreover, the specific characteristics of the 16S rDNA sequence of CCUG 19559 infer that up to one-third of the gene may have been acquired from *C. jejuni* in a horizontal gene transfer event, a phenomenon that has attracted substantial credence in recent years (30). However, the fact that most of the *C. upsaliensis* and *C. helveticus* strains tested did not give an amplicon in the assay described indicates that the selectivity is acceptable.

Since the assay may be considered as an ISO or European international standard for detection of thermotolerant *Campy-*

lobacter in food, it was of considerable importance to find the best DNA polymerase enzyme for the assay: i.e., that most resistant to PCR inhibitors naturally occurring in foods and chicken samples in particular. We evaluated DyNAyme, T*th*, and *Taq* for their ability to withstand inhibitors from chicken rinse. To facilitate the evaluation of the effect of the sample matrix only, regardless of the specificity of the selected *Campylobacter* primers, an already validated PCR assay for detection of pathogenic *Y. enterocolitica* (14) was used as a test model.

The results indicated T*th* to be the DNA polymerase of choice when examining chicken wash samples, since the PCR was substantially less inhibited when this enzyme was used compared with the *Taq* and DynaZyme polymerases. The improved performance of the PCR assay by use of T*th* polymerase was observed in both the conventional and real-time PCR assays studied, which we consider to be an important observation. Real-time PCR assays are becoming of increasing importance in food quality matters, since they assess the level of contamination (and not simply the presence or absence) with a given pathogen. Based on the results obtained in chicken rinse, T*th* DNA polymerase was chosen for international validation of the selected PCR assay with the highest specificity. This assay employs a novel combination of two previously published primers, the forward primer OT1559 (5) and the reverse primer 18-1 (25), and amplifies a 287-bp sequence of the 16S gene.

We conclude that the PCR test designed in the present study could form the basis of an accurate, standardized, and robust high-throughput, screening tool for enteropathogenic campylobacters in foods. Results from an international collaborative trial are described elsewhere (16).

ACKNOWLEDGMENTS

The work was supported by EU project no. QLK1-CT-1999-00226 (FOOD-PCR).

We thank Jeanette Knudsen, Lise Christensen, Kirsten Vestergaard, and Penny Jordan for excellent technical assistance; Eva Møller Nielsen for providing us with the field strains; Mathilde H. Josefsen and Nigel Cook for critical reading of the manuscript; and Stefan Jensen for editorial work.

REFERENCES

1. **Abu Al-Soud, W., and P. Rådström.** 1998. Capacity of nine thermostable DNA polymerases to mediate DNA amplification in the presence of PCR-inhibiting samples. Appl. Environ. Microbiol. **64:**3748–3753.
2. **Angen, Ø., P. Ahrens, and C. Tegtmeier.** 1998. Development of a species-specific PCR test for identification of *Haemophilus somnus* in pure and mixed cultures. Vet. Microbiol. **63:**39–48.
3. **Anonymous.** 2002. Microbiology of food and animal feeding stuffs—protocol for the validation of alternative methods (EN ISO/FIDS 16140). European Committee for Standardization, AFNOR, Paris, France.
4. **Burnett, T. A., M. A. Hornitzky, P. Kuhnert, and S. P. Djordjevic.** 2002. Speciating *Campylobacter jejuni* and *Campylobacter coli* isolates from poultry and humans using six PCR-based assays. FEMS Microbiol. Lett. **216:**201–209.
5. **Docherty, L., M. R. Adams, P. Patel, and J. McFadden.** 1996. The magnetic immuno-polymerase chain reaction assay for the detection of *Campylobacter* in milk and poultry. Lett. Appl. Microbiol. **22:**288–292.
6. **Fermér, C., and E. O. Engvall.** 1999. Specific PCR identification and differentiation of the thermophilic campylobacters, *Campylobacter jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis.* J. Clin. Microbiol. **37:**3370–3373.
7. **Freidman, C. R., J. Neimann, H. C. Wegener, and R. V. Tauxe.** 2000. Epidemiology of *Campylobacter* jejuni infections in the United States and other industrialized nations, p. 121–138. *In* I. Nachamkin and M. J. Blaser (ed.), *Campylobacter*, 2nd ed. ASM Press, Washington, D.C.
8. **Goossens, H., and J.-P. Butzler.** 1992. Isolation and identification of *Campylobacter* spp., p. 93–109. *In* I. Nachamkin, M. J. Blaser, and L. S. Tompkins (ed.), *Campylobacter jejuni.* Current status and future trends. ASM Press, Washington, D.C.
9. **Hanai, K., M. Satake, H. Nakanishi, and K. Venkateswaran.** 1997. Comparison of commercially available kits with standard methods for detection of *Salmonella* strains in foods. Appl. Environ. Microbiol. **63:**775–778.
10. **Hoorfar, J., and N. Cook.** 2002. Critical steps in standardization of PCR. Methods Mol. Biol. **216:**51–64.
11. **Hoorfar, J., P. Ahrens, and P. Rådström.** 2000. Automated 5′ nuclease PCR assay for identification of *Salmonella enterica*. J. Clin. Microbiol. **38:**3429–3435.
12. **Jacobs-Reitsma, W.** 2000. *Campylobacter* in the food supply, p. 467–481. *In* I. Nachamkin and M. J. Blaser (ed.), *Campylobacter*, 2nd ed. ASM Press, Washington, D.C.
13. **Josefsen, M., P. S. Lübeck, B. Aalbæk, and J. Hoorfar.** 2002. Preston and Park-Sanders protocols adapted for semi-quantitative isolation of thermotolerant *Campylobacter* from chicken rinse. Int. J. Food Microbiol. **80:**177–183.
14. **Lantz, P. G., R. Knutsson, Y. Blixt, W. Abu Al-Soud, E. Borch, and P. Rådström.** 1998. Detection of pathogenic *Yersinia enterocolitica* in enrichment media and pork by a multiplex PCR: a study of sample preparation and PCR-inhibitory components. Int. J. Food Microbiol. **45:**93–105.
15. **Lübeck, P. S. and J. Hoorfar.** 2002. PCR technology and applications to zoonotic food-borne bacterial pathogens. Methods Mol. Biol. **216:**65–83.
16. **Lübeck, P. S., N. Cook, M. Wagner, P. Fach, and J. Hoorfar.** 2003. Towards an international standard for PCR-based detection of foodborne thermotolerant campylobacters: validation in multi-center collaborative trials. Appl. Environ. Microbiol. **69:**5670–5672.
17. **Malorny, B., P. T. Tassios, P. Rådström, N. Cook, M. Wagner, and J. Hoorfar.** 2003. Standardization of diagnostic PCR for the detection of foodborne pathogens. Int. J. Food. Microbiol. **83:**39–48.
18. **Moorhead, S. M., and G. A. Dykes.** 2002. Survival of *Campylobacter jejuni* on beef trimmings during freezing and frozen storage. Lett. Appl. Microbiol. **34:**72–76.
19. **On, S. L. W.** 1996. Identification methods for campylobacters, helicobacters, and related organisms. Clin. Microbiol. Rev. **9:**405–422.
20. **On, S. L. W.** 2001. Taxonomy of *Campylobacter*, *Arcobacter*, *Helicobacter*, and related bacteria: current status, future prospects, and immediate concerns. Symp. Suppl. Soc. Appl. Microbiol. **90:**1S–15S.
21. **On, S. L. W., 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.
22. **Park, R. W. A., P. L. Griffiths, and G. S. Moreno.** 1991. Sources and survival of campylobacters: relevance to enteritis and the food industry. Symp. Suppl. J. Appl. Bacteriol. **70:**97S–106S.
23. **Scheu, P. M., K. Berghof, and U. Stahl.** 1998. Detection of pathogenic and spoilage microorganisms in food with the polymerase chain reaction. Food Microbiol. **15:**13–31.
24. **Trust, T. J., S. M. Logan, C. E. Gustafson, P. J. Romaniuk, N. W. Kim, V. L. Chan, M. A. Ragan, P. Guerry, and R. R. Gutell.** 1994. Phylogenetic and molecular characterization of a 23S rRNA gene positions the genus *Campylobacter* in the epsilon subdivision of the *Proteobacteria* and shows that the presence of transcribed spacers is common in *Campylobacter* spp. J. Bacteriol. **176:**4597–4609.
25. **Uyttendaele, M., R. Schukkink, B. van Gemen, and J. Debevere.** 1994. Identification of *Campylobacter jejuni*, *Campylobacter coli* and *Campylobacter lari* by the nucleic acid amplification system NASBA. J. Appl. Bacteriol. **77:**694–701.
26. **Van Berkum, P., Z. Terefework, L. Paulin, S. Suomalainen, K. Lindström, and B. D. Eardly.** 2003. Discordant phylogenies within the *rrn* loci of rhizobia. J. Bacteriol. **185:**2988–2998.
27. **Vaneechoutte, M., and J. Van Eldere.** 1997. The possibilities and limitations of nucleic acid amplification technology in diagnostic microbiology. J. Med. Microbiol. **46:**188–194.
28. **Vanniasinkam, T., J. A. Lanser, and M. D. Barton.** 1999. PCR for the detection of *Campylobacter* spp. in clinical specimens. Lett. Appl. Microbiol. **28:**52–56.
29. **Waller, D. F., and S. A. Ogata.** 2000. Quantitative immunocapture PCR assay for detection of *Campylobacter jejuni* in foods. Appl. Environ. Microbiol. **66:**4115–4118.
30. **Young, J. M.** 2001. Implications of alternative classifications and horizontal gene transfer for bacterial taxonomy. Int. J. Sys. Evol. Microbiol. **51:**945–953.