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Development of real-time PCR and hybridization methods for detection and identification of thermophilic *Campylobacter* spp. in pig faecal samples

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ABSTRACT

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Aims: To develop a real-time (rt) PCR for species differentiation of thermophilic *Campylobacter* and to develop a method for assessing co-colonization of pigs by *Campylobacter* spp.

Methods and Results: The specificity of a developed 5′ nuclease rt-PCR for species-specific identification of *Campylobacter jejuni*, *Campylobacter coli*, *Campylobacter lari*, *Campylobacter upsaliensis* and of a *hipO* gene nucleotide probe for detection of *C. jejuni* by colony-blot hybridization were determined by testing a total of 75 reference strains of *Campylobacter* spp. and related organisms. The rt-PCR method allowed species-specific detection of *Campylobacter* spp. in naturally infected pig faecal samples after an enrichment step, whereas the hybridization approach enhanced the specific isolation of *C. jejuni* (present in minority to *C. coli*) from pigs.

Conclusions: The rt-PCR was specific for *Campylobacter jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis* and the colony-blot hybridization approach provided an effective tool for isolation of *C. jejuni* from pig faecal samples typically dominated by *C. coli*.

Significance and Impact of the Study: Species differentiation between thermophilic *Campylobacter* is difficult by phenotypic methods and the developed rt-PCR provides an easy and fast method for such differentiation. Detection of *C. jejuni* by colony hybridization may increase the isolation rate of this species from pig faeces.

Keywords: *Campylobacter jejuni*, *hipO* gene, hybridization, identification, pigs, real-time PCR, thermophilic *Campylobacter*.

INTRODUCTION

Campylobacter jejuni is the dominant cause of human campylobacteriosis. This dominance of *C. jejuni* is also found in most healthy carrier animals, for example, broiler chickens, cattle and wild-living birds and mammals (Nielsen *et al.* 1997; Petersen *et al.* 2001). In contrast, most studies on conventional pigs show a dominance of *Campylobacter coli* often with colonization of several *Campylobacter* serotypes in the same pig (Munroe *et al.* 1983; Manser and Dalziel 1985; Weijtens *et al.* 1999), although an American study found a high occurrence of *C. jejuni* (Young *et al.* 2000). *Campylobacter jejuni* may also coexist with *C. coli*, but are typically present in 10–100-fold lower numbers than *C. coli*. Hence, *C. jejuni* will be less likely isolated from such samples, because typically only a few colonies are identified to the species level with conventional culturing and biochemical testing techniques.

Correct differentiation of thermophilic *Campylobacter* spp. by phenotypic tests is difficult (Steinhauserova *et al.* 2001), and can be problematic if only one character distinguishes the two species. The use of hippurate hydrolysis, which is the only specific characteristic that differentiates *C. jejuni* from *C. coli*, is problematic, because hippurate-variable or hippurate-negative strains have been reported (Totten *et al.*

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1987; Wainø *et al.* 2003). Hence, hippurate hydrolysis-negative *C. jejuni* strains may be misclassified as *C. coli*.

Nucleic acid-based methods provide good alternatives for identification of *Campylobacter* and in particular, PCR was widely applied for identification and detection of *Campylobacter* spp. (e.g. Linton *et al.* 1997; Denis *et al.* 1999; Burnett *et al.* 2002; Cloak and Fratamico 2002; Lübeck *et al.* 2003; On and Jordan 2003). Development of real-time PCR (rt-PCR) assays is preferable to conventional PCR because of the increased specificity, sensitivity and possible quantitative approach. In addition, there is a reduced risk of cross-contamination of the closed-tube system of rt-PCR, requiring no further handling of the products opposed to gel electrophoresis of conventional PCR. Despite these advantages of the rt-PCR technique, its usage for a molecular identification of *C. coli*, *C. jejuni*, *Campylobacter lari* and *Campylobacter upsaliensis* at species level has not yet been reported. Recently, rt-PCR for identification of *Campylobacter jejuni* and/or *C. coli* isolates (Nogva *et al.* 2000; Wilson *et al.* 2000; Best *et al.* 2003) and detection of *C. jejuni* in naturally contaminated samples such as foods (Sails *et al.* 2003), poultry, milk and water (Yang *et al.* 2003), cattle faeces (Inglis and Kalischuk 2004) and chicken cecum/faeces (Rudi *et al.* 2004) have been reported. An alternative two-step rt-PCR method reported by Logan *et al.* (2001) differentiated by melting peak analysis between different *Campylobacter* spp. but not between *C. jejuni* and *C. coli*.

Application of rt-PCR may show the presence of specific bacteria or gene sequences, however, it is still required to obtain bacterial isolates for further elucidation of the diversity among strains by serotyping and genotyping such as restriction fragment length polymorphism.

Therefore, one of our aims in this study was to develop a rt-PCR method for species-specific identification of isolates of *C. coli*, *C. jejuni*, *C. lari* and *C. upsaliensis*. This rt-PCR method was also examined for its ability to detect *C. jejuni* among predominant *C. coli* in naturally infected enriched pig faecal samples for an assessment of co-colonization of *Campylobacter* species in pigs. Another aim was to develop a method for specific detection of *C. jejuni* colonies in mixed populations as an alternative to random selection of *Campylobacter* spp. used in conventional methods. For this purpose, a nonradioactive labelled colony-blot hybridization method for detection of *C. jejuni* colonies from pig faecal samples including the ability to obtain *C. jejuni* isolates was evaluated.

MATERIALS AND METHODS

Bacterial strains

A total of 75 different *Campylobacter* spp. and related *Helicobacter*, *Wollinella* and *Arcobacter* reference strains from the CCUG, ATCC or NCTC culture collections were used for testing the specificity of primers and probes for rt-PCR identification of *C. jejuni*, *C. coli*, *C. lari* and *C. upsaliensis* and for evaluation of *C. jejuni* colony detection by colony-blot hybridization with a digoxigenin (DIG)-labelled probe (Table 1). In addition, 29 hippurate hydrolysis-negative but presumptive *C. jejuni* isolates (human and pig origin) from our inhouse collection were included (Table 1). *Campylobacter jejuni*, *C. coli*, *C. lari*, *C. upsaliensis*, *Campylobacter concisus*, *Campylobacter hyointestinalis*, *Campylobacter showae* and *Campylobacter mucosalis* were grown at 41·5°C for 48 h in microaerobic atmosphere on calf blood agar (BA) plates (Statens Serum Institut, Denmark). *Arcobacter skirrowii* and *Arcobacter cryaerophilus* were grown under similar conditions but at 30°C. *Arcobacter nitrofigilis* was grown at 25°C for 48 h in microaerobic atmosphere on BA plates containing 2% NaCl. *Campylobacter curvus*, *Campylobacter gracilis*, *Campylobacter rectus* and *Wollinella succinogens* were grown anaerobically at 37°C for 48–72 h. The remaining strains were incubated at 37°C for 48 h in microaerobic atmosphere on BA plates (Table 1).

Hippurate hydrolysis test

A loopful of cell material (*c.* 10 μl) from BA plates was added to 0·4-ml sterile filtered 1% sodium hippurate solution and incubated at 37°C for 2 h after which 0·2-ml 3·5% ninhydrin solution dissolved in acetone–butanol (1 : 1 v/v) was added. The appearance of a purple colour reaction within 10 min of incubation at 37°C was denoted as a positive result.

Real-time PCR for differentiation of thermophilic *Campylobacter*

One loopful (*c.* 10 μl) of colony material from pure bacterial cultures on BA plates was suspended in 200 μl of sterile destilled water and lysed in a heat block at 100°C for 10 min. Lysates were centrifuged (4°C) at 10 000 *g* for 3 min to pellet cell debris and the supernatant was used for rt-PCR analysis.

Species-specific primers and probes for rt-PCR (5′ nuclease) identification of *C. jejuni*, *C. coli*, *C. lari* and *C. upsaliensis* were designed based on the nucleotide sequence of the *glyA* gene, which encodes serine hydroxymethyltransferase, of *C. jejuni* ATCC 33560, *C. coli* ATCC 33559, *C. lari* ATCC 35221 and *C. upsaliensis* ATCC43954 (GenBank accession numbers AF136493–AF136496) (Table 2) (Al Rashid *et al.* 2000). This gene is highly conserved but shows enough sequence variation to allow differentiation between these four *Campylobacter* species. The *Campylobacter glyA* sequences were further aligned to the *glyA* sequences of the closely related *Arcobacter butzleri*

Table 1 Evaluation and comparison of real-time-PCR (*glyA*), DIG probe colony-blot hybridization (*hipO*) and hippurate hydrolysis by testing *Campylobacter* ssp. and related organisms

Bacterial species (*n*)	Name of strain	*glyA* gene rt-PCR identification	*HipO* gene probe hybridization	Hydrolysis of hippurate
C. jejuni ssp. *jejuni* ($n = 23$)	CCUG 11284,CCUG 26272, NCTC 11392, P1, P2, P3, P4, P5j, P6, P7, P8, P11, P15, P18, P19, P21, P23, P27, P33, P42, P44, P55, P57	*C. jejuni*	Positive	Positive
C. jejuni ssp. *doylei*	CCUG 24567	*C. jejuni*	Positive	Positive
C. coli ($n = 21$)	CCUG 11283, ATCC 33559, CCUG 33450, CCUG 11352, P5c, P14, P20, P24, P25, P26, P28, P30, P34, P39, P46, P48, P49, P54, P56, P59, P61	*C. coli*	Negative	Negative
C. lari ($n = 4$)	CCUG 23947, CCUG 18267, CCUG 20575, CCUG 19512	*C. lari*	Negative	Negative
C. upsaliensis ($n = 7$)	CCUG 23626, CCUG 14913, CCUG 19559, CCUG 33890, CCUG 20818, CCUG 24571, CCUG 24803	*C. upsaliensis*	Negative	Negative
C. mucosalis	CCUG 6822	Negative	Negative	Negative
C. hyointestinalis	CCUG 14169	Negative	Negative	Negative
C. concisus	CCUG 13144	Negative	Negative	Negative
C. fetus ssp. *fetus*	CCUG 6823	Negative	Negative	Negative
C. showae	CCUG 30254	Negative	Negative	Negative
C. helveticus	CCUG 30682	Negative	Negative	Negative
C. curvus	CCUG 13146	Negative	Negative	Negative
C. gracilis	CCUG 27720	Negative	Negative	Negative
C. rectus	CCUG 20446†	Negative	Negative	ND
C. lanienae	NCTC 13004†	Negative	Negative	ND
Helicobacter pylori	CCUG 17874	Negative	Negative	Negative
H. hepaticus	CCUG 33637	Negative	ND	Negative
H. fennellieae	CCUG 18820T	Negative	ND	Negative
Arcobacter butzleri ($n = 2$)	CCUG 30485, CCUG 10373	Negative	Negative	Negative
A. skirrowii	CCUG 10374	Negative	Negative	Negative
A. cryaerophilus	CCUG 17801	Negative	Negative	Negative
A. nitrofigilis	CCUG 15893	Negative	Negative	ND
Wolinella succinogenes	CCUG 13145T	Negative	Negative	ND
Human isolates ($n = 4$)	WS011270, WS011166, S20123, S20275	*C. jejuni*	Positive	Negative
Pig isolates ($n = 25$)	1–25	*C. jejuni*	Positive	Negative

n, number of strains.

NCTC, National Collection of Type Cultures, (Colindale, UK); CCUG, Culture Collection, University of Göteborg (Göteborg, Sweden); ATCC, American Type Culture Collection (Manassas, Va); T typestrain and P 'Penner scheme' serostrains (CCUG reference strains) based on heat-stable (HS) antigens (Penner and Hennessey 1980).

†hybridization was performed on a DNA boil lysate instead of colony material.

(ATCC46916 and ATCC13218) to avoid regions with sequence homology for design of primers and probes. Primer Express Software (version 2·0; Applied Biosystems, Foster City, CA, USA) together with the corresponding guidelines (User's Manual; Applied Biosystems) was used in the design of the primers and probes. Primers and probes were run through a BLAST search, which showed that the sequences were species specific. The oligonucleotides used were synthesized by DNA Technology (Aarhus, Denmark) (Table 2).

Table 2 Probes and primers used for automated 5′ nuclease PCR assay (*glyA* gene)

Species (size in bp)	Primer or probe name	Primer and probe sequences 5′ → 3′	Reporter dye*
C. coli (80)	Cc-F	GTTGGAGCTTATCTTTTTGCAGACA	
	Cc-R	TGAGGAAATGGACTTGGATGCT	
	Cc-P	TGCTACAACAAGTCCAGCAATGTGTGCA	TET
C. jejuni (135)	Cj-F	TAATGTTCAGCCTAATTCAGGTTCTC	
	Cj-R	GAAGAACTTACTTTTGCACCATGAGT	
	Cj-P	AATCAAAGCCGCATAAACACCTTGATTAGC	FAM
C. lari (96)	Cl-F	CAGGCTTGGTTGTAGCAGGTG	
	Cl-R	ACCCCTTGGACCTCTTAAAGTTTT	
	Cl-P	CATCCTAGTCCATTCCCTTATGCTCATGTT	TET
C. upsaliensis (65)	Cu-F	TCGTAGCTGGTGAGCATCCTAG	
	Cu-R	GGTTTTGTGTGTGGTTGAGCTT	
	Cu-P	CCTTTCCCTCACGCACACATCG	FAM

bp, length in base pairs of the species-specific PCR products.
*The probes were dual-labelled with either FAM (6-carboxyfluorescein) or TET (tetrachloro-6-carboxy-flurorescein) on the 5′-end, and quenched by 6-carboxy-tetramethyl-rhodamine (TAMRA) at the 3′-end.

The rt-PCR was carried out for each *Campylobacter* spp. (single-plex) in a 20-μl PCR reaction volume containing TaqMan Universal Mastermix (Applied Biosystems), sterile destilled water, 600 nmol l^{-1} each primer, 200 nmol l^{-1} each probe and 2 μl of template DNA prepared as described above. The rt-PCR was performed in the ABI Prism 7700 sequence detection system (Applied Biosystems) with thermocycler conditions and postPCR analysis as described by Nielsen and Andersen (2003).

HipO gene nucleotide probe for detection of *C. jejuni* colonies

A primer-pair termed HIP400F and HIP1134R for specific amplification of *C. jejuni* (735-bp sized amplicon) was previously designed on basis of the sequence of the *N*-benzoylglycine amidohydrolase hippuricase (*hipO*) gene, which is absent from *Campylobacter* spp. other than *C. jejuni* (Hani and Chan 1995; Linton *et al.* 1997). These primers were used (1 μmol l^{-1} each) to prepare a *C. jejuni*-specific *hipO* gene nucleotide probe labelled with DIG by incorporating DIG-11-dUTP using the PCR DIG Probe Synthesis Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's recommendations. A 2-μl of DNA from pure cultures was prepared as described above from *C. jejuni* CCUG 11284, *C. jejuni* 'Penner scheme' serotype strains 2, 4, 6, 11, 23 and 35, respectively, and used as template DNA in the preparation of probes. The PCR thermocycler conditions were 94°C for 5 min; 30 cycles of 1 min at 94°C, 1 min at 62°C and 2 min at 72°C and final extension for 8 min at 72°C.

The specificity of the *hipO* gene probe for detection of *C. jejuni* by colony-blot hybridization was evaluated with the 73 *Campylobacter* spp. and related organisms listed in Table 1. Cell material from single colonies of these strains were inoculated in the pattern of a grid onto two BA plates successively using a 1-μl loop, and incubated as appropriate for 24–48 h, followed by colony-blot hybridization according to the guidelines of the user's manual (Roche Diagnostics). The DNA was cross-linked to the membranes by exposure to UV illumination for 3 min and membranes were prehybridized at 60°C for 1 h before hybridization overnight at 60°C with a mixture of the prepared denatured *hipO* gene nucleotide probes (2 μl probe solution per ml of hybridization buffer). The DIG-labelled probe-target hybrids were detected using alkaline phosphatase conjugated anti-DIG antibody and 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/4-nitroblue-tetrazolium chloride (NBT) as colour substrate according to the manufacturers recommendations (Roche Diagnostics). Development of a purple colour reaction on the membranes was denoted as hybridization positive colonies (*C. jejuni*).

Detection of *C. jejuni* in pig faecal samples

A total of 261 rectal faecal samples from 7 to 13-week-old pigs were examined within 24 h after collection, for the presence of *C. jejuni*. Four different methods were compared for the ability to detect the presence of *C. jejuni* in pig faecal samples, (i) analysing enriched samples (Bolton broth without blood, prepared according to the recommendations of the 'Bacteriological Analytical Manual Online'; Hunt *et al.* 1998) by rt-PCR, (ii) colony-blot hybridization with the *hipO* gene probe, either on (ii-a) enriched sample (referred to as enriched hybridization) or (ii-b) nonenriched

sample (referred to as *direct hybridization*), and finally by (iii) random picking of *Campylobacter* colonies (see flow chart in Fig. 1).

Microbiological method. One gram of faecal material was suspended in 9 ml of Bolton broth, 10-fold dilution series (10^{-1}–10^{-4}) were prepared and 0·1 ml of each dilution was inoculated onto modified charcoal-cefoperazone-deoxycholate agar plates (CCDA) [*Campylobacter* Blood-Free Selective Agar Base (Oxoid, Basingstoke, UK) with CCDA Selective Supplement (SE155E; Oxoid)] for enumeration, isolation and colony-blot hybridization. The enrichment broth dilutions (a total of 134 samples for rt-PCR screening and enriched hybridization) and CCDA plates (a total of 261 samples for enumeration and direct hybridization) were incubated for 48 h at 41·5°C in a microaerobic atmosphere (Fig. 1).

Rt-PCR screening of enriched faecal samples. The enriched sample dilutions were analysed for the presence of *C. jejuni* and *C. coli* by the rt-PCR assay described above. For this, template DNA was prepared by centrifugation of 1 ml of the enrichment broth at 10 000 *g* for 5 min. Then, the pellet was washed in 1 ml 0·9% NaCl and re-suspended in 50 μl of modified TE buffer (10 mmol l^{-1} Tris–HCl, 0·1 mmol l^{-1} EDTA, pH 7·6) after centrifugation. Finally, the cells were lysed and analysed in the rt-PCR assay as described for pure cultures.

Although quantification is possible with rt-PCR, this rt-PCR was used to determine if *C. jejuni* and *C. coli* were present (qualitatively) in the enriched sample dilutions. Generally, samples with cycle threshold (C_t) >30 and clear indication of probe cleavage (judged by the multicomponent analysis) were considered positive as described by Nielsen and Andersen (2003).

Colony-blot hybridization on enriched samples. In case an enriched faecal sample was detected *C. jejuni* positive in rt-PCR, the sample was subjected to analysis by colony-blot hybridization to detect *C. jejuni* and to possibly obtain bacterial isolates by the enriched hybridization method described earlier. As agar plates with single colonies were preferable for optimal colony hybridization, *C. jejuni*-positive enrichment broth dilutions were further 10-fold diluted in 0·85% saline solution with 0·1% peptone before inoculation onto CCDA plates. After 48 h of incubation, the CCDA plate with most single colonies was selected and the membrane discs and plates were marked for orientation before performing colony-blot hybridization as described above.

Colony-blot hybridization on nonenriched samples. Direct hybridization was applied for detection of *C. jejuni* colonies as a simpler alternative to the enriched hybridization. Thus, the CCDA plates inoculated with the dilution series of nonenriched faecal material (261 samples for enumeration of *Campylobacter* spp.) were subjected to

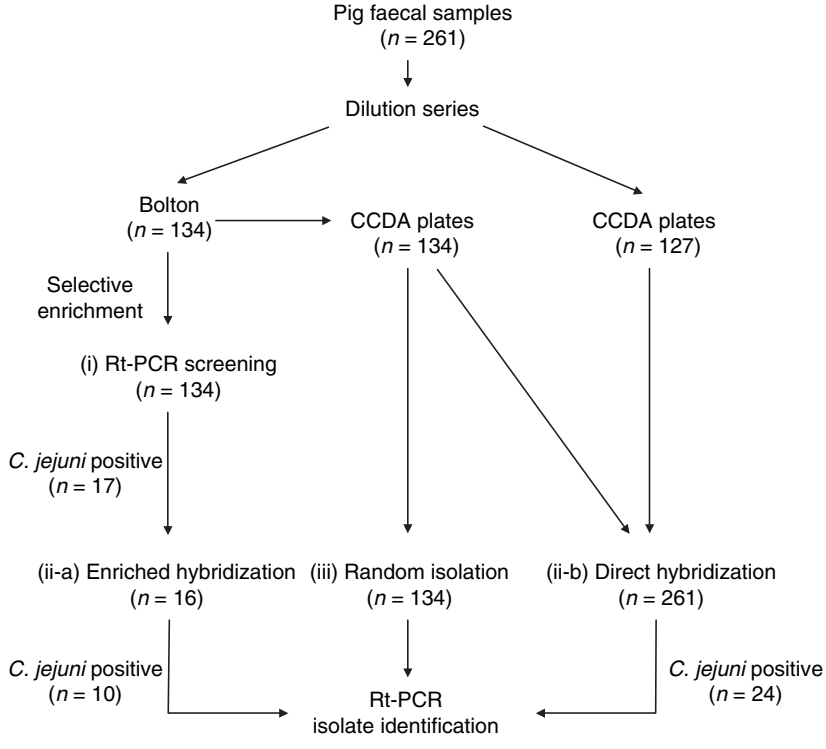

Fig. 1 Overview and flow charts of the comparison of four different methods for detection of *Campylobacter jejuni* in pig faecal samples

colony-blot hybridization (direct) with no prior screening of the *C. jejuni* status.

Bacterial isolates. When probe-target hybrids (presumptive *C. jejuni* colonies) were detected by hybridization, cell material from the corresponding colony on the CCDA plate (reincubated for 48 h after the colony lift) was picked and subcultured by restreaking single colonies twice onto new BA plates in an attempt to obtain pure cultures.

The specific detection of *C. jejuni* was compared with 'random' isolation of *Campylobacter* spp. These 'random' isolates were obtained from five presumptive *Campyloacter* spp. colonies (based on colony and cell morphology) on the CCDA plates inoculated with nonenriched pig faecal samples (used for direct hybridization). Colonies were only obtained from those faecal samples that were subjected to both direct hybridization and rt-PCR (enriched hybridization) ($n = 134$) (Fig. 1). The isolates were selected to represent the different colony morphologies present on each plate. The presumptive *C. jejuni* isolates obtained from hybridization and the 'random' *Campylobacter* spp. isolates were all subjected to the hippurate hydrolysis test and the rt-PCR assay for identification.

RESULTS

Evaluation of real-time PCR detection of *Campylobacter* spp.

A total of 23 *C. jejuni* ssp. *jejuni*, one *C. jejuni* ssp. *doylei*, 21 *C. coli*, seven *C. upsaliensis*, and four *C. lari* were correctly amplified by single-plex rt-PCR with no cross reactions between the four species (Table 1). In addition, only negative results were obtained when 10 other *Campylobacter* spp., five *Arcobacter* strains, one *Wolinella* strain and three *Helicobacter* species were tested in the rt-PCR test to evaluate the specificity of the primers and probe.

Evaluation of the *hipO* gene probe for detection of *Campylobacter jejuni*

The colony-blot hybridization of 24 *C. jejuni* strains with the DIG-labelled *hipO* gene nucleotide probe yielded clearly purple probe-target hybrids after chromogenic detection (Table 1). However, the detection of *C. jejuni* Penner strains 2 and 11 was sometimes less distinct. An increase of the denaturation time for the bacterial cells while blotting onto the membrane (according to user's manual) from 5 to 15 min enhanced the signal of the Penner strains 2 and 11 (data not given). Slater and Owen (1997) showed that the hippuricase gene is highly conserved in Penner heat-stable serotypes 1, 4 and 11. In addition, 42 *Campylobacter* non-*jejuni* and seven closely related non-*Campylobacter* strains yielded no target-probe hybrids, which indicated a good specificity of the *hipO* probe.

Detection of *C. jejuni* in pig faecal samples

Four methods were compared for the ability to detect *C. jejuni* in pig faecal samples: including screening of enrichment broth by real-time PCR; *hipO* gene probe colony-blot hybridization either on enriched or nonenriched (direct) samples; and finally by random picking of *Campylobacter* colonies. The rt-PCR on enrichment cultures revealed the presence of *C. coli* in all samples and *C. jejuni* in 17 of the 134 samples (Table 3). Thus, the developed of rt-PCR method for differentiation of thermophilic *Campylobacter* spp. enabled specific detection of *C. coli* and *C. jejuni* in mixed populations of naturally infected pig faecal samples after an enrichment step.

Comparison of rt-PCR and colony-blot hybridization. Identification of *C. jejuni* colonies by colony-blot hybridization showed that most, but not all 17 samples, which contained *C. jejuni* as shown by rt-PCR, were found

Table 3 Comparison of the rt-PCR results (no. of samples*) with colony-blot hybridization and random isolation for detection of *Campylobacter jejuni* in pig faecal samples

	Colony-blot hybridization (DIG-labelled probe-*hipO* gene)							
	Enrichment followed by hybridization			Direct hybridization			Random isolation	
Result of rt-PCR screening (n)	Positive	Negative	ND	Positive	Negative	ND	Positive	Negative
C. jejuni-positive $n = 17$	10†	6	1	8	9	0	5	12
C. jejuni-negative $n = 117$			117	3‡	101	13	1	116

n, number of faecal samples.

*Positive, *C. jejuni* positive; negative, *C. jejuni* negative; ND, not done.

†Bacterial *C. jejuni* isolates only confirmed from eight samples.

‡Bacterial isolates only obtained from one sample.

to yield purple colonies by the two colony hybridization approaches (Table 3). The two different hybridization approaches gave the same result in nine (five negative, four positive) of the 16 comparable samples with neither of the methods being superior to the other. However, the CFU ratio between *C. jejuni* (target-probe hybrids) and total *Campylobacter* ssp. seemed to be higher for the enriched samples with 635 of 1612 CFU (enriched hybridization) compared with 166 of 1274 CFU for the nonenriched samples (direct hybridization).

The direct hybridization approach was performed on 134 samples and detected target-probe hybrids (*C. jejuni*) in three of the 104 samples found *C. jejuni*-negative by rt-PCR (hybridization was not feasible for the remaining 13 samples that were *C. jejuni*-negative by rt-PCR because the colony density was too high) (Table 3). However, confirmation of *C. jejuni* by isolation from the presumptive *C. jejuni* colonies was only performed for one of the three samples. In addition, the direct hybridization approach was applied to 127 samples as the single *C. jejuni* detection method, which further indicated the ability of the *hipO* gene probe to detect *C. jejuni* colonies in minority to *Campylobacter* ssp. (99 of 2335 CFU) in 13 cases.

Identification of bacterial isolates. A total of 117 isolates (representing 27 samples) were picked to confirm the identity of the presumptive *C. jejuni* colonies detected by either direct or enriched hybridization. Eighty-nine strains were confirmed to be *C. jejuni* by rt-PCR, whereas 17 were *C. coli*, one was neither *C. coli* nor *C. jejuni*, and 10 colonies failed for unknown reasons to show growth. In 10 cases, the filter membranes showed some vaguely coloured target-probe hybrids (referred to as suspect), but the isolates obtained from these were identified as *C. coli* by the rt-PCR method. The presumptive *C. jejuni* isolates identified as non-*C. jejuni* in the rt-PCR test, were retested by colony-blot hybridization with the *hipO* probe. All these strains were negative by hybridization in agreement with the rt-PCR identification. Hence, the non-*C. jejuni* isolates obtained from the presumptive *C. jejuni* colonies were apparently not because of poor specificity of the *hipO* gene probe. Instead, the problem may have been to isolate the right colony or colonies of mixed strains, where the restreaking of colony material in an attempt to obtain pure cultures may have lead to a loss of *C. jejuni*.

The isolates were also tested for their ability to hydrolyse hippurate, which is a specific characteristic for *C. jejuni*, normally used for phenotypic discrimination from other *Campylobacter* spp. In this study, the development of colour indicating hydrolysis of hippurate either failed or was very weak in two repeated tests of 25 isolates, varied for 30 isolates and was positive for 44 isolates of 89 isolates. This occurred despite they were all identified as *C. jejuni* by rt-PCR test and the *hipO* gene probe (Table 1).

Random isolation of isolates. Finally, the specific detection of *C. jejuni* by rt-PCR or colony hybridization was compared with isolation of *Campylobacter* spp. by picking of five random colonies from each sample. Rt-PCR identification of the obtained isolates showed that six samples were *C. jejuni* positives with 18 of the 560 isolates being *C. jejuni* (Table 3). *Campylobacter jejuni* was also obtained from these six samples by at least one of the hybridization methods, but rt-PCR screening of the enriched faecal samples failed to detect the presence in one of these samples.

DISCUSSION

The developed 5′ nuclease rt-PCR based on the *glyA* gene correctly identified representative reference culture strains of *C. jejuni*, *C. coli*, *C. lari* and *C. upsaliensis* in pure cultures, providing a good and easy alternative to the often inadequate microbiological phenotypic methods for differentiation between these species. Furthermore, the rt-PCR test also enabled the detection of *C. jejuni* and *C. coli* present in pig faecal samples enriched for 2 d in Bolton broth. This indicates that the enrichment in Bolton broth support the growth of *C. jejuni* even when present in lower numbers than *C. coli*. This was also indicated by an increased ratio of hybridization target-probe hybrids (*C. jejuni*) to total *Campylobacter* CFU, when samples that were enriched were compared with nonenriched samples (direct hybridization). Nevertheless, enrichment of *C. jejuni* in pig faecal samples can be difficult due to overgrowth by the more numerous *C. coli* and a high background flora in general (Corry *et al.* 1995; Madden *et al.* 2000). This may explain the detection of *C. jejuni* in three nonenriched samples either by direct hybridization (three samples) or random isolation (one sample), while the enrichments of these samples were found *C. jejuni* negative by rt-PCR. As *C. coli* was amplified in all the enriched faecal samples, it was unlikely that the prepared DNA could have been inhibitory to the rt-PCR enzyme reaction and thereby leading to false negatives.

The rt-PCR screening enabled the detection of *Campylobacter* spp. in enriched faecal samples and the differentiation between *C. coli* and *C. jejuni*. Thus, rt-PCR can detect a possible co-colonization of *Campylobacter* species, which may be missed by conventional methods because of the often low numbers of *C. jejuni* in pigs. However, the applied boiling method for lysis of bacterial cells may have biased the detection because of the existence of *Campylobacter* strains resistant to lysis by boiling that would then be undetectable by PCR (Englen and Kelly 2000). A comparison of rt-PCR screening results based on DNA prepared by different methods would have addressed this.

The specific rt-PCR detection of *C. jejuni* can be applied as a single method, but isolation of bacterial strains for

further characterization would be valuable, for example, to study diversity of *Campylobacter* spp. from pigs by serotyping and genotyping (Weijtens *et al.* 1999).

This study showed that the developed DIG-labelled nucleotide probe based on the hippuricase (*hipO*) gene in combination with either enriched or direct colony-blot hybridization provided a tool for specific identification and isolation of *C. jejuni* present in low numbers in mixed populations of pig faecal samples. The 17 bacterial isolates not confirmed as *C. jejuni* (*C. coli*) could probably be explained by difficulties in identifying the correct colony or overgrowth from neighbouring colonies rather than a poor specificity of the *hipO* nucleotide probe, as rehybridization of the isolates was negative. Other reports on *Campylobacter* detection by colony blot hybridization did not provide the opportunity of obtaining bacterial isolates for subsequent characterization (Taylor and Hiratsuka 1990; Ng *et al.* 1997). Furthermore, our PCR DIG-labelling of a specific nucleotide sequence by use of specific primers for the hippuricase gene provided a more specific and easy method compared with the cloning approach described by Taylor and Hiratsuka (1990).

When comparing the two different hybridization approaches, only nine samples were found to yield the same hybridization results and four of these were hybridization negative. Furthermore, with target-probe hybrids (*C. jejuni*) found in 10 and eight samples for enriched and direct hybridization, respectively, neither of the approaches seemed to be superior to the other. Hence, the preferable approach may depend on the expected frequency of *C. jejuni*, for example, rt-PCR screening will reduce the number of hybridizations if only few samples contain *C. jejuni* but instead require preparations of DNA and rt-PCR analysis. Further, pre-enrichment may be necessary to obtain colonies for hybridization in the case of a low level of *Campylobacter*.

It was assumed that only the most predominant species was likely to be chosen by the conventional method with random picking of a few colonies. However, when five isolates were obtained from each of 134 pig faecal samples, *C. jejuni* was found in six cases, compared with 10 and 11 for the two hybridization methods. Isolation of five colonies with different morphology instead of only one colony as in normal procedure may have enhanced the likelihood of obtaining *C. jejuni* so the beneficence of specific detection by hybridization became less distinct.

The prevalence of hippurate-negative *C. jejuni* in this study was surprisingly high, as detection of *C. jejuni* by the ability to hydrolyse hippurate only, would have misclassified 25 (28%) rt-PCR *C. jejuni* isolates as *C. coli*. Whereas, Totten *et al.* (1987) and Wainø *et al.* (2003) found that hippurate-negative *C. jejuni* represented 1·6 and 13·4%, respectively, of *C. jejuni* strains obtained from human patients and chickens, respectively. An additional 30 (33%) strains showed a variable result in two testings and this variation occurred despite a standard method being applied. These results emphasize the need for nonphenotypic method alternatives, as the rt-PCR test described.

The hippurate-hydrolysis negative *C. jejuni* were detected by the *hipO* nucleotide probe, which was based on the hippuricase gene sequence. This indicates the presence of this gene and its absence is therefore not a likely explanation of the hippurate-hydrolysis negative strains, which is in agreement with the studies by Hani and Chan (1995) and Slater and Owen (1997). However, the cause of the hydrolyse activity defect has not yet been elucidated.

In conclusion, this study shows that the developed rt-PCR assays provide easy and fast differentiation of *C. jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis*. Furthermore, the colony-blot hybridization approach provided a tool for isolation of *C. jejuni* from pig faecal samples with a *Campylobacter* population that was dominated by *C. coli*.

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