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Note

Identification of *Campylobacter fetus* by fluorescence *in situ* hybridization (FISH)

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ABSTRACT

Two new DNA FISH-probes for *Campylobacter fetus* were designed, *in silico* checked for cross-reactions and successfully evaluated in a multi-centric approach with 41 *Campylobacter fetus* isolates including isolates of all three know subspecies: *Campylobacter fetus* ssp. *fetus*, *Campylobacter fetus* ssp. *venerealis*, and *Campylobacter fetus* ssp. *testudinum* and 40 strains of five non-target *Campylobacter* species.

The bacterial species *Campylobacter fetus* is divided into three different subspecies. *C. fetus* ssp. *fetus* is commonly associated with enzootic abortion especially in sheep and bacteremia in humans (Abbass et al. 2011), whereas *C. fetus* ssp. *venerealis* typically causes bovine genital campylobacteriosis causing infertility with considerable economic losses (van Bergen et al. 2005; Wagenaar et al. 2014). A third subspecies – *C. fetus* ssp. *testudinum* – has been isolated from various reptile species, which is a colonizer in these hosts. *C. fetus* ssp. *testudinum* was also associated with bacteremia in humans (Dingle et al. 2010; Fitzgerald et al. 2014).

Fluorescence *in situ* hybridization (FISH) is one of various methods established for molecular identification of *Campylobacter* spp. A FISH protocol using a hierarchical probe set for the rapid discrimination of isolates of thermotolerant *Campylobacter* spp. after growth in culture has been established for the diagnostic (Poppert et al. 2008). However, *Campylobacter fetus* could not be included due to a lack of well-characterized isolates as positive controls. To close this gap *C. fetus* specific FISH probes were designed and evaluated with a panel of well-characterized isolates in a multi-centric approach.

A total of 20*C. fetus* spp. *fetus*, 14*C. fetus* spp. *venerealis*, 7*C. fetus* spp. *testudinum* isolates, 10*C. jejuni* spp. *jejuni*, 4*C. jejuni* ssp. *doylei*, 14*C. coli*, 10*C. lari*, and 2*C. upsaliensis* isolates were included for the probe evaluation. The *C. fetus* isolates originated from bull preputial washings (n = 16), cow vaginal mucus (n = 2), aborted calf fetuses (n = 4), aborted lamb fetus (n = 1), intestine of swine (n = 1), intestine of calf (n = 1), colonized reptiles (n = 3), and human blood samples (n = 11). Details are listed in Supplementary Tables 1–4.

Two sulfoindocyanine-(Cy3-)labeled DNA probes for FISH targeting 16S rRNA of *Campylobacter fetus* (*C_fetus*_1: 5′- Cy3-GAG-ATT-AGT-TGG-ATA-TCA-AGC-CC-3′ and *C_fetus*_16s_1027: 5′-Cy3-CCT-GTC-TCA-ACT-TTC-TAG-CAA-GC-3) were designed using the ARB software (Kumar et al. 2006; Ludwig et al. 2004). Afterwards, they were evaluated *in silico* for cross-binding using the probeCheck software (http://www.microbial-ecology.net/probecheck) (Loy et al. 2008) referring to the SILVA LSU reference database (Pruesse et al. 2007). Probes were synthesized by Sequence Laboratories (Seqlab) Göttingen GmbH, Göttingen, Germany.

Optimal hybridization conditions for the new *C. fetus* probes were assessed by increasing the formamide concentration in the hybridization buffer at 46 °C from 10% to 50% as previously described (Frickmann et al. 2017; Moter and Göbel 2000).

Campylobacter FISH was performed as described before (Poppert

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et al. 2008) with minor modifications: bacterial suspensions originating from Mueller-Hinton-Broth cultures were directly transferred to the slides. Fixation of specimens was done using methanol (10 min.) instead of paraformaldehyde and the slides were dried in ambient air. *C. fetus* probes were used in combination with the fluorescein-(FAM-)labeled eubacterial probe EUB338 (Amann et al. 1990) as an internal control. The hybridization process was performed in a closed FISH-Hybrite chamber (Vysis HYBrite, Abbott Diagnostics, Abbott Park, Illinois, USA) at 46 °C for 90 min. The slides were placed in a washing buffer at 38 °C for 20 min (Poppert et al. 2008). Counterstaining of bacterial DNA was performed with 4′,6-diamidino-2-phenylindole (DAPI) included in the commercial mounting medium Vectashield (Vector Laboratories, Inc., Burlingame,USA). Staining with FISH probes of each bacterial isolate was done in duplicate at three different occasions.

In addition, a series of experiments with spiked blood culture bottles was carried out to simulate the situation of potential *C. fetus*/non-*C. fetus* bacteraemia. For this purpose, 24 BACT/ALERT™ blood culture bottles (bioMérieux Deutschland GmbH, Nürtingen, Germany; 12 aerobic and 12 anaerobic bottles) were filled with 20 mL of human packed red blood cells (kindly provided by the Department for Transfusion Medicine of the University Medical Center Göttingen) and inoculated with 12*C. fetus* (4*C. fetus* ssp. *fetus*, 4C. *fetus* ssp. *venerealis*, 4*C. fetus* ssp. *testudinum*) and 12 non-*C. fetus* (4*C. jejuni* ssp. *jejuni*, 4*C. coli*, 2*C. lari*, 2*C. upsaliensis*) isolates (one *Campylobacter* isolate per bottle). A volume of 0.5 mL of a *Campylobacter* suspension with an OD_{600} = 0.5 in Mueller-Hinton broth was used for inoculation. The inoculated blood culture bottles were incubated for 24 h at 37 °C. Thereafter, FISH was performed as described above.

In silico evaluation of the *C. fetus* FISH probes using probeCheck confirmed concordance of both probes with deposited *C. fetus* sequence information. Thereby, the *C_fetus*_16s_1027 probe showed matches in the one-base-pair mismatch range with sequence data of non-*C. fetus Campylobacter* species indicating a higher specificity of a test using the *C_fetus*_1 probe when compared to *C_fetus*_16s_1027. Details are shown in Table 1.

The hybridization optimum of the newly designed FISH probes was confirmed at 38% formamide. The C_fetus_1 probe showed signals with 37/41 (90.2% sensitivity) of the *C. fetus* strains but did not cross-react with the 40 non-*C. fetus Campylobacter* isolates used as negative controls (100% specificity). One of 20*C. fetus* ssp. fetus isolates and three of 14*C. fetus* ssp. *venerealis* isolates were consistently not detected in all three replications. In contrast, the sensitivity for *C. fetus* ssp. *testudinum* isolates was 100% (n = 7).

The C_fetus_16s_1027 probe showed a cross-reaction with one *C. coli* isolate of the negative control samples (1/14). No cross-reactions were observed in the other non-*C. fetus Campylobacter* species: *C. jejuni* ssp. *jejuni* (0/10), *C. jejuni* ssp. *doylei* (0/4), *C. lari* (0/10), and *C. upsaliensis* (0/2). This gives a specificity of 97.5% (1/40) for the probe C_fetus_16s_1027. Positive results were obtained with all tested *C. fetus* isolates of all three *C. fetus* subspecies (sensitivity 100%; Fig. 1). Details are shown in Table 2.

When testing both probes in spiked blood culture bottles, only a single sample spiked with a *C. fetus* ssp. *venerealis* isolate showed a borderline result consistently in all three replications when tested for the C_fetus_1 probe. Here a weak fluorescence hard at the decision boundary between positive and negative was detected. In the end, we decided to rate this sample as negative. Thus, the C_fetus_1 probe had a sensitivity of 91.6% (11/12) and a specificity of 100% (0/12), while sensitivity and specificity of the C_fetus_16s_1027 probe were 100% (12/12 and 0/12) in spiked blood culture bottles.

The evaluation of the two newly designed *C. fetus* FISH probes showed good sensitivity with slightly reduced specificity for probe C_fetus_16S_1027. These results suggest its use for screening. The probe C_fetus_1, in contrast, showed reduced sensitivity but good specificity in the *in vitro* evaluation with the non-*C. fetus* species: *C. jejuni* spp. *jejuni*, *C. jejuni* ssp. *doylei*, *C. coli*, *C. lari*, and *C. upsaliensis*. Therefore, it is more suitable for confirmation testing. *In silico* alignment studies have disclosed that there are non-target species that have only one-base-pair or two-base-pairs mismatches, which may pose a risk for cross-reactions. Nevertheless, the close phylogenetic relationship within the genus *Campylobacter* suggests the combined use of both *C. fetus* probes in the proposed screening-and-confirmation approach to ensure satisfactory specificity.

The evaluated *C. fetus* probes provide a useful amendment to the previously introduced panel of diagnostic FISH probes for *Campylobacter* spp. (Poppert et al. 2008). All three subspecies of *C. fetus* can cause human disease presenting with bacteremia and sepsis especially in immunocompromised patients but also endocarditis, meningitis or enteritis can be seen in the immunocompetent host (Abbass et al. 2011; Fujihara et al. 2006; Inoue et al. 1993; Montero et al. 1997; Peterson 1994, p. 199; Sauerwein et al. 1993; Wagenaar et al. 2014). Even sporadic cases of *C. fetus* ssp. *testudinum* bacteremia in humans have been reported (Fitzgerald et al. 2014).

FISH based on cost-efficient DNA probes, which are not protected by patents, is considerably less expensive than using patent-protected and commercially available nucleic acid-mimicking peptide-nucleic-acid (PNA) probes (Lehtola et al. 2005). Cost effectiveness is an issue for research use of molecular diagnostic approaches if the available funding is scarce. The costs per reaction for consumables for FISH using non-patent-protected DNA probes are less than 1 USD/1 Euro (Frickmann et al. 2017). Thus, in-house FISH is considerably cheaper than the most amplification-based molecular diagnostic techniques.

Competing interests

The authors have declared that no competing interests exist.

Table 1
In silico alignment studies of the C. fetus probes “C. fetus 1” and “C. fetus 16s 1027”.

Mis-match count	Ribosomal RNA sequence corresponding to the probe	Matching with known sequences (no. of entries)
Probe *C. fetus* 1: 5′-GAG ATT AGT TGG ATA TCA AGC CC-3’		
0	GGG-CUU-GAU-AUC-CAA-CUA-AUC-UC	*Campylobacter fetus* (15), *Campylobacter fetus* ssp. *fetus* (16), *Campylobacter fetus* ssp. *venerealis* (7)
2	GGG-CUU-GAU-AUC-CAA-**UG**A-AUC-UC	uncultured bacterium (5)
	AGG-CUU-GAU-AUC-CAA-C**A**A-AUC-UC	*Campylobacter hyointestinalis (1) uncultured rumen bacterium (8)*
Probe *C. fetus* 16 s 1027: 5’-CCT GTC TCA ACT TTC TAG CAA GC -3‘		
0	GCU-UGC-UAG-AAA-GUU-GAG-ACA-GG	*Campylobacter fetus* (15), *Campylobacter fetus* ssp. *fetus* (17), *Campylobacter fetus* ssp. *venerealis* (7), *Campylobacter avium* (3)
1	GCU-UGC-UAG-AA**U**-GUU-GAG-ACA-GG	*Campylobacter upsaliensis* (20), *Campylobacter mucosalis* (8), *Campylobacter hyointestinalis* ssp. *hyointestinalis* (1), *Campylobacter helveticus* (1), *Campylobacter troglodytis* (3), uncultured bacterium (4), uncultured rumen bacterium (10), *Campylobacter mucosalis*-like-bacterium (1)
	GCU-UGC-UAG-AA**C**-GUU-GAG-ACA-GG	*Campylobacter sp.* 0402576-C0066 (1), *Campylobacter sp.* 0401536-C0027 (1)
	GCU-UGC-UAG-A**gC**-GUU-GAG-ACA-GG	uncultured bacterium (1)
2	GCU-UGC-UAG-AAA-GUU-GAG-A**GC**-GG	uncultured bacterium (2), uncultured marine bacterium (2)