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Evaluation of molecular assays for identification *Campylobacter fetus* species and subspecies and development of a *C. fetus* specific real-time PCR assay

Linda van der Graaf-van Bloois [a,c], Marcel A.P. van Bergen [b,c], Fimme J. van der Wal [b,c], Albert G. de Boer [b,c], Birgitta Duim [a,c], Tracy Schmidt [d], Jaap A. Wagenaar [a,b,c,*]

[a] *Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, P.O. Box 80165, 3508 TD Utrecht, The Netherlands*
[b] *Central Veterinary Institute of Wageningen UR, P.O. Box 65, 8200 AB Lelystad, The Netherlands*
[c] *WHO Collaborating Center for Campylobacter and OIE Reference Laboratory for Campylobacteriosis, The Netherlands*
[d] *Allerton Provincial Veterinary Laboratory, Private Bag X2, Cascades, 3202, South Africa*

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ABSTRACT

Phenotypic differentiation between *Campylobacter fetus* (*C. fetus*) subspecies *fetus* and *C. fetus* subspecies *venerealis* is hampered by poor reliability and reproducibility of biochemical assays. AFLP (amplified fragment length polymorphism) and MLST (multilocus sequence typing) are the molecular standards for *C. fetus* subspecies identification, but these methods are laborious and expensive. Several PCR assays for *C. fetus* subspecies identification have been described, but a reliable comparison of these assays is lacking.

The aim of this study was to evaluate the most practical and routinely implementable published PCR assays designed for *C. fetus* species and subspecies identification. The sensitivity and specificity of the assays were calculated by using an extensively characterized and diverse collection of *C. fetus* strains. AFLP and MLST identification were used as reference. Two PCR assays were able to identify *C. fetus* strains correctly at species level. The *C. fetus* species identification target, gene *nahE*, of one PCR assay was used to develop a real-time PCR assay with 100% sensitivity and 100% specificity, but the development of a subspecies *venerealis* specific real-time PCR (IS*fe1*) failed due to sequence variation of the target insertion sequence and prevalence in other *Campylobacter* species. None of the published PCR assays was able to identify *C. fetus* strains correctly at subspecies level. Molecular analysis by AFLP or MLST is still recommended to identify *C. fetus* isolates at subspecies level.

1. Introduction

Bovine genital campylobacteriosis (BGC) is a syndrome characterized by fertility problems in cattle (Dekeyser, 1984). The causative agent of BGC is *Campylobacter fetus* subspecies *venerealis* (Cfv). Cfv is restricted to the genital tract of both male and female cattle and includes a variant, Cfv biovar *intermedius* (Cfvi) (Véron and Chatelain, 1973). This subspecies is venereally transmitted only (Garcia et al., 1983). The other *C. fetus* subspecies is *Campylobacter fetus* subspecies *fetus* (Cff), which occurs mainly in the intestinal tract of cattle and sheep (Garcia et al., 1983). There are three transmission routes for this subspecies: fecal–oral, ascending genital infections most probably due to intestinal colonization, and venereal transmission (Véron and Chatelain, 1973; Garcia et al., 1983). Cff can cause sporadic infections in humans, abortion in cattle and sheep, and is incidentally isolated from a variety of sites in different hosts (Véron and Chatelain, 1973; Garcia et al., 1983).

A criterion for an effective BGC control program is the reliable differentiation of *C. fetus* subspecies *venerealis* from the closely related *C. fetus* subspecies *fetus*. The World Animal Health Organisation (OIE) Terrestrial Code (OIE, 2013b) describes the health measures to be taken by the veterinary authorities. In addition to these standards and recommendations, countries can have their local or regional regulatory measures as well, for example the regulations of the European Union (http://eur-lex.europa.eu/nl/index.htm). Several countries have been successful in eradicating BGC, whereas in many other countries BGC is still endemic (OIE, 2013a). Due to trade of livestock, individual cases and outbreaks with Cfv are occasionally detected in countries with the BGC-free status. Depending on the regulations concerning BGC, there are severe consequences for artificial insemination (AI) stations when a Cfv positive animal is identified: temporary closure and destruction of semen that was produced since the last negative monitoring results. Misidentification of Cfv as Cff may result into the spread of Cfv into cattle populations. Misidentification of Cff as Cfv may result in economic losses. Taken together, the consequences of subspecies

* Corresponding author at: Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, P.O. Box 80165, 3508 TD Utrecht, The Netherlands. Tel.: +31 30 2534376; fax: +31 30 2533199.
E-mail address: j.wagenaar@uu.nl (J.A. Wagenaar).

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misidentification can be severe, showing that indisputably *C. fetus* subspecies identification is crucial for BGC control programs.

The two *C. fetus* subspecies can be differentiated phenotypically by the 1% glycine tolerance test (Véron and Chatelain, 1973), but this test has poor reproducibility (van Bergen et al., 2005a). The hydrogen sulphide (H_2S) test is also described to differentiate Cff (H_2S positive) from Cfv (H_2S negative) (Véron and Chatelain, 1973). However, as Cfv biovar *intermedius* strains are positive in the H_2S test, this assay is not suitable to differentiate between the two subspecies (van Bergen et al., 2005a). To overcome these problems, molecular assays for subspecies differentiation have been introduced.

In literature, several molecular assays for *C. fetus* species and subspecies identification have been described that are laborious or not suitable for standard routine diagnostic laboratories; hybridization assays (Wesley et al., 1991; Blom et al., 1995; Casademont et al., 2000), restriction fragment length polymorphism (RFLP) (Eaglesome et al., 1995; Cardarelli-Leite et al., 1996), 16S sequencing (Gorkiewicz et al., 2003) and pulsed field gel electrophoresis (PFGE) (Salama et al., 1992; Vargas et al., 2003). More practical assays for identification have been described, based on nucleic acid amplification such as nested PCR, targeting 16S and 23S rRNA (Inglis and Kalischuk, 2003) and loop-mediated isothermal amplification (LAMP) for *C. fetus* species and subspecies *venerealis* detection (Yamazaki et al., 2009; Yamazaki et al., 2010). However, nested PCR is not preferred for routine diagnostics, since this type of assay is sensitive for contamination, whereas LAMP is relatively unknown and uncommon in diagnostic laboratories; importantly, the target of this LAMP assay (insertion element IS*fe1*) is not suitable for differentiation between the two *C. fetus* subspecies, as shown in this study. The molecular typing methods amplified fragment length polymorphism (AFLP) (Wagenaar et al., 2001) and multilocus sequence typing (MLST) (van Bergen et al., 2005a) proved to be able to differentiate the two subspecies reliably and can be used for identification of species and subspecies, but these tests are laborious and impractical for routine use.

A preferred platform for routine diagnostics is PCR, since this type of assay is fast, easy and reliable. A number of PCR assays have been developed to identify *C. fetus* species. A PCR assay targeting 16S rRNA (Oyarzabal et al., 1997) differentiated *C. fetus* from other *Campylobacter* species, but only a single base mismatch in the target 16S rRNA region differentiates the two *C. fetus* subspecies, making this region too conserved to enable successful differentiation of the two subspecies (Oyarzabal et al., 1997). A PCR assay targeting 23S rRNA identifies *C. fetus* species, but attempts to design primers on this target to discriminate between the two subspecies failed (Bastyns et al., 1994). PCR assays targeting the *cdt*-genes (Asakura et al., 2007) and *cpn60*-gene (Chaban et al., 2009) are described for *C. fetus* species identification, but these assays are not able to discriminate between the two subspecies. Since subspecies *venerealis* is described as the causative agent of BGC, it is very important for routine diagnostic laboratories to differentiate between the two subspecies and assays that only identify *C. fetus* to the species level are not sufficient. Lastly, two subspecies *venerealis* specific PCRs have been described, targeting virulence genes (Moolhuijzen et al., 2009; Iraola et al., 2012). However, Gorkiewicz et al. (2010) showed that these virulence genes are not present in all Cfv strains and are therefore not suitable for subspecies *venerealis* identification.

For this study, PCR assays that were reported to be able to differentiate the two subspecies were selected (Table 1). The multiplex PCR assay of Hum et al. (1997) is included to evaluate the suitability of the target genes *cstA* and *parA*, therefore the modified PCR assays using the same targets are excluded (Muller et al., 2003; Schulze et al., 2006; Schmidt et al., 2010; Iraola et al., 2012; Chaban et al., 2012). Since McMillen et al. (2006) used real-time PCR instead of conventional PCR, this assay is included in this study.

The first goal of this study was to evaluate the selected PCR assays for both species and subspecies identification of *C. fetus*. The sensitivity and specificity of the assays were calculated by using an extensively characterized collection of strains from different sources and geographical regions. The second goal of this study was to improve *C. fetus* diagnostics. Based on the evaluation of published PCR assays, the gene *nahE* was used to develop a real-time PCR assay for *C. fetus* species identification and insertion sequence IS*fe1* for subspecies *venerealis* identification.

Table 1
Evaluated PCR assays.

Code	Reference	Identification	Target genes	Assay
A	Abril et al. (2007)	*C. fetus* Cfv	*nahE* IS*fe1*	Multiplex PCR
B	van Bergen et al. (2005b)	Cfv	Hypothetical protein	PCR
H	Hum et al. (1997)	*C. fetus* Cfv	*cstA* *parA*	Multiplex PCR
M	McMillen et al. (2006)	Cfv	*parA*	Real-time PCR
W	Wang et al. (2002)	Cff	*sapB2*	PCR
	Tu et al. (2005)	Cff/Cfv	Not applicable	RAPD–PCR

2. Materials and methods

2.1. Strains

The PCR assays were tested with a collection of 143 *C. fetus* strains (83 Cff and 60 Cfv) (Supplemental Table 1) from different sources and geographical regions. Twelve non-*fetus Campylobacter* reference strains (Supplemental Table 1) were included to verify the specificity of the assays; these strains represent non-*fetus Campylobacter* species that are occasionally isolated from bovine or ovine samples.

Strains were grown for 48 h on heart-infusion agar supplemented with 5% sheep blood (Biotrading, Mijdrecht, The Netherlands) under micro-aerobic conditions (6% O_2, 7% CO_2, 7% H_2, 80% N_2, Anoxomat, Mart Microbiology, Lichtenvoorde, The Netherlands). Chromosomal DNA of all strains was isolated with the PureGene kit (Gentra PureGene DNA isolation kit, Qiagen). PCR A (see Section 2.2) and the new real-time PCR assay (*nahE*) (see Section 2.4) were also tested with boiled cell lysates. These boiled cell lysates were made by suspending cells ($OD_{600\ nm} = 0.1$) in sterile distilled water and subsequent heating at 95 °C for 10 min.

All *C. fetus* and non-*fetus Campylobacter* strains were characterized by AFLP as described (Wagenaar et al., 2001). The *C. fetus* strains were also characterized by phenotypic assays, i.e. growth in presence of 1% glycine and H_2S production, and MLST (van Bergen et al., 2005a).

2.2. PCR assays

The PCR assays, labeled A, B, H, and W (Table 1) were performed with the thermal cycling programs as described in the original publications (Hum et al., 1997; Wang et al., 2002; Tu et al., 2005; van Bergen et al., 2005b; Abril et al., 2007) with the following reaction-mixture modifications: PCR assays were performed with 2.5 U AmpliTaq DNA polymerase (Applied Biosystems), the supplied PCR buffer II and 2.5 mM $MgCl_2$. PCR H was performed with 2.5 U AmpliTaq DNA polymerase (Applied Biosystems) and PCR buffer as described in the original publication. All reactions were performed in a GeneAmp PCR System 9700 thermocycler (Applied Biosystems). Real-time PCR M (McMillen et al., 2006) was performed with the thermal cycling program as described in the original publication in a 7500 Fast Real-Time PCR System (Applied Biosystems), by using 2× TaqMan Fast Universal PCR Master Mix (Applied Biosystems) with primers, probe and DNA concentration as described in the original publication.

The new real-time PCR assays, described in Section 2.4, were performed in 20 μl on a 7500 Fast Real-Time PCR System (Applied Biosystems) in Fast 7500 run mode, using the following thermal cycler protocol: 95 °C for 20 s, followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s. Each PCR reaction contained 10 μl 2× TaqMan Fast Universal

Table 2
Real-time PCR primers and probes for *C. fetus* species and subspecies *venerealis* identification.

Name	Sequence (5′ → 3′)	Function
nahE-F	TGTTATGGTGATCAAAATAGCTGTTG	Forward primer
nahE-R	GAGCTGTTTTTATGGCTACTCTTTTTTTA	Reverse primer
nahE-P	*FAM*-TGTATATGCACTTTTAGCAACTT-*NFQ*	NFQ-MGB probe
ISC1-F	AGGCGAAGAGAATGTTAAATTTGAA	Forward primer
ISC1-R	CCATAAAGCCTAGCTGAAAAAACTG	Reverse primer
ISC1-P	*VIC*-CCAAAGATGTCTTAGAAATA-*NFQ*	NFQ-MGB probe
ISC2-F	TTCAAAAGCTCTTGGGGTTAC	Forward primer
ISC2-R	AAAGCCTTGTTTAGAACAATATAACTC	Reverse primer
ISC2-P	*VIC*-ACTCGTGGTGGAGAGCGTAG-*NFQ*	NFQ-MGB probe

PCR Master Mix (Applied Biosystems), 1 μl (12 pmol/μl) of each primer, 0.5 μl (8 pmol/μl) probe, and 2 μl of either DNA template or cell lysate, adjusted to 20 μl with nuclease free-water. Using a threshold of 0.1, a result was designated positive for Ct's below 30.

2.3. Sequencing of insertion sequence ISCfe1

The sequence of IS*Cfe1* of Cfv strain NCTC 10354 (GenBank AM260752.1) was used to design primers with Primer Express 3.0 (Applied Biosystems), outside the Cfv specific target region of PCR A (Abril et al., 2007). The resulting fragment, containing IS*Cfe1*, was sequenced for one Cfv strain (B27) using Sanger sequencing. Roche 454 sequence data of 16 Cfv strains (manuscript in preparation) was used to BLAST the insertion sequences, with the sequence of IS*Cfe1* of Cfv strain NCTC 10354 as scaffold. The IS sequences, obtained with BLAST analysis from whole genome sequences of 16 Cfv strains (including Zaf 3) and the IS*Cfe1* sequence of strain B27, were aligned and compared to serve as target for designing a new real-time PCR.

2.4. New real-time PCR assay for species and subspecies identification

To develop a new real-time PCR assay, the *C. fetus* species specific target gene *nahE* and the subspecies *venerealis* specific insertion sequence (IS) IS*Cfe1* (Abril et al., 2007) were used. With Primer Express 3.0 (Applied Biosystems), primers and a Taqman NFQ-MGB probe were designed for *C. fetus* species identification based on gene *nahE* (Table 2). For subspecies *venerealis* identification, real-time primers ISC1-F, ISC1-R and probe ISC1-P (Table 2) were developed within IS*Cfe1* from bp 585 to 668. Within the conserved region of the IS, a second set of primers ISC2-F, ISC2-R and probe ISC2-P (Table 2) were developed, covering the region from bp 132 to 387. The program and reaction mixtures for the new real time PCRs are described in Section 2.2.

3. Results

3.1. PCR assays

One published RAPD–PCR assay (Tu et al., 2005) was excluded from the evaluation as a preliminary evaluation of this assay yielded a very low sensitivity with only 3/7 Cff and 3/8 Cfv strains correctly identified (data not shown).

The results of all evaluated PCR assays with 143 *C. fetus* strains (83 Cff and 60 Cfv) and 12 non-*fetus Campylobacter* strains are shown in Supplemental Table 1. The specificity and sensitivity and the 95% confidence intervals (CIs) (Dohoo et al., 2003) of the assays were calculated, using AFLP and MLST subspecies identification as reference (Table 3).

Two PCR assays showed 100% sensitivity and 100% specificity for *C. fetus* species identification, PCR A (Abril et al., 2007) and PCR H (Hum et al., 1997). None of the evaluated PCR assays was able to identify all *C. fetus* strains correctly at subspecies level. For subspecies identification, PCR A showed the highest sensitivity (97%) and specificity (100%). Only two Cfv strains, B27 and Zaf 3, were misidentified as Cff with this PCR. To investigate this deviation, the IS*Cfe1* sequences of these strains were determined (see Section 3.2).

For multiplex PCR A (Abril et al., 2007) and the newly developed *C. fetus* specific real-time PCR (*nahE*), the results using purified DNA were identical to PCR results using boiled cell lysates (data not shown).

3.2. Sequencing of insertion sequence ISCfe1

Alignment of the sequences to the IS*Cfe1* sequence of strain NCTC 10354 indicated the existence of at least two different types of insertion sequences, ISa and ISb, sharing 98.7% sequence homology. BLAST analysis indicated that all Cfv strains contained different copy numbers of these insertion sequences, where the majority contained ISa and two strains exclusively contained ISb (B27 and Zaf3) (Table 4). One of the Cfv specific primers of PCR A, primer Cven-L (Abril et al., 2007), is located on a region where the sequences of ISa and ISb differ. The sequence differences result in five mismatches of primer Cven-L with ISb, which are likely to affect the misidentification of PCR A for Cfv strains B27 and Zaf3.

Table 3
Estimated sensitivity and specificity of the PCR assays with AFLP and MLST subspecies identification as reference.

Assay	Identification	Sensitivity		(95% CI)	Specificity		(95% CI)
Abril (A)	*C. fetus*	143/143[a]	100%	(97–100)	12/12[d]	100%	(76–100)
	Cfv	58/60[b]	97%	(87–99)	95/95[e]	100%	(96–100)
van Bergen (B)	Cfv	27/60[b]	45%	(33–57)	95/95[e]	100%	(96–100)
Hum (H)	*C. fetus*	143/143[a]	100%	(97–100)	12/12[d]	100%	(76–100)
	Cfv	35/60[b]	58%	(46–70)	79/95[e]	83%	(74–89)
McMillen (M)	Cfv	32/60[b]	53%	(41–65)	95/95[e]	100%	(96–100)
Wang (W)	Cff	63/83[c]	76%	(66–84)	52/72[f]	72%	(61–81)
This study:							
Real-time PCR (*nahE*)	*C. fetus*	143/143[a]	100%	(97–100)	12/12[d]	100%	(76–100)
Real-time PCR (IS*Cfe1*) – *ISC1*	Cfv	58/60[a]	97%	(88–99)	95/95[c]	100%	(96–100)
Real-time PCR (IS*Cfe1*) – *ISC2*	Cfv	60/60[a]	100%	(94–100)	93/95[c]	98%	(93–99)

[a] Number of *C. fetus* strains: 143.
[b] Number of *C. fetus* subsp. *venerealis* strains: 60.
[c] Number of *C. fetus* subsp. *fetus* strains: 83.
[d] Number of non-*fetus Campylobacter* strains: 12.
[e] Number of non-Cfv strains: 95 (83[c] + 12[d]).
[f] Number of non-Cff strains: 72 (60[b] + 12[d]).

Table 4
Estimated copy numbers of insertion sequences in genome sequences.

Strain	Subspecies	Source	Country	Copy number ISa	Copy number ISb
97/608	Cfv	Bovine	AR	7	1
84/112	Cfv	Bovine	USA	3	–
B10	Cfv	Bovine	US	5	–
B27	Cfv	Bovine	US	–	≥1*
CCUG 33872	Cfv	Unknown	CZ	3	–
CCUG 33900	Cfv	Bovine	FR	5	–
LMG 6570	Cfv	Bovine	BE	3	–
Zaf 3	Cfv	Bovine	ZA	–	2
01/165	Cfvi	Bovine	AR	6	–
02/298	Cfvi	Bovine	AR	4	–
03/293	Cfvi	Bovine	AR	6	–
03/596	Cfvi	Bovine	AR	5	–
92/203	Cfvi	Bovine	AR	7	–
97/532	Cfvi	Bovine	AR	7	–
98/25	Cfvi	Bovine	AR	5	–
WBT 011/09	Cfvi	Unknown	UK	5	–
Zaf 65	Cfvi	Bovine	ZA	4	–

* Data obtained by Sanger sequencing, estimation of copy numbers of IS in genome is not possible.

3.3. Real-time PCR assay for species and subspecies identification

The newly developed real-time PCR assay for *C. fetus* species identification, targeting gene *nahE*, performed excellently with 100% sensitivity and 100% specificity (Table 3). For subspecies *venerealis* identification, real-time primers and probe (ISC1) targeting the same region of IS*Cfe1* as PCR A, resulted in misidentification of two Cfv strains (B27 and Zaf 3) as Cff. An alternative approach with primers and a probe (ISC2), targeting the conserved region of the IS, failed as two *Campylobacter hyointestinalis* reference strains were misidentified as subspecies *venerealis*. It has not been possible to develop primers on target IS*Cfe1* that result in 100% sensitivity and 100% specificity for subspecies *venerealis* identification.

4. Discussion

In this study, the molecular typing assays AFLP and MLST were used as reference to evaluate PCR assays for *C. fetus* species and subspecies identification, since AFLP and MLST proved to be able to differentiate the two subspecies reliably. AFLP analysis is a reliable typing method to differentiate between Cff, Cfv and Cfvi. The MLST housekeeping gene sequences of *C. fetus* are very stable (van Bergen et al., 2005a), which makes MLST a very robust typing method. Nevertheless, the resolution is slightly lower as Cfv and Cfvi strains cannot be distinguished by MLST, since they belong to the same sequence type (ST), i.e. ST-4, ST-7 and ST-12. When the MLST result of a *C. fetus* isolate indicates an existing ST, the subspecies can be reliably identified, but when a new ST is found, the subspecies cannot be identified and AFLP needs to be performed (van Bergen et al., 2005a).

PCR A (Abril et al., 2007) is a multiplex PCR assay, designed to simultaneously identify strains for *C. fetus* species and subspecies *venerealis*. The *C. fetus* species target of this PCR is gene *nahE*. PCR A was able to identify *C. fetus* strains correctly at species level. For subspecies *venerealis* identification, this PCR targets the insertion sequence IS*Cfe1*, which is present in Cfv, but absent in Cff. It was shown in this study that at least two different ISs (ISa and ISb) can occur in subspecies *venerealis* strains. In two Cfv strains containing only ISb, the *venerealis* specific primers were not able to amplify the target IS. The fact that strains from different continents (Africa and North America) carry only ISb, shows that the occurrence of the ISb variant does not depend on the geographic origin of the strains. This result also stresses the importance of inclusion of isolates from different geographical origins when an assay is validated for global use. The inability to identify all Cfv strains correctly renders this PCR unsuitable for subtyping *C. fetus* strains.

PCR B (van Bergen et al., 2005b) was designed to identify subspecies *venerealis*. With PCR B, all Cfv sensu stricto strains were correctly identified, but the assay did not detect any of the Cfv biovar *intermedius* strains. Therefore, PCR B is not suitable for diagnostic purposes. If, in the future, a reliable PCR assay becomes available that detects all Cfv including biovar *intermedius* strains, PCR B may be used as an additional epidemiological tool to distinguish Cfv sensu stricto strains from biovar *intermedius* strains.

PCR H (Hum et al., 1997) is a multiplex PCR assay, designed to identify *C. fetus* species and subspecies *venerealis*. The *C. fetus* species identification of this PCR, based on target gene *cstA*, performs excellently. For subspecies *venerealis* identification, PCR H and PCR M (McMillen et al., 2006) use the same target, gene *parA*. The target gene *parA* can be located on a transferable genomic island or on a plasmid, and strains can contain multiple copy numbers of this gene (Abril et al., 2010; Gorkiewicz et al., 2010). The sensitivity and specificity of the Cfv subspecies identification of PCR H and PCR M with target *parA* are poor, confirming the results from a previous publication (Willoughby et al., 2005). In a recent study, PCR M showed a positive result with a *C. hyointestinalis* strain isolated from a bull (Spence et al., 2011). This observation and the low sensitivity observed in this study render the *parA* gene and consequently all other PCR assays using this target (Muller et al., 2003; Schulze et al., 2006; Schmidt et al., 2010; Iraola et al., 2012; Chaban et al., 2012) unsuitable for Cfv identification.

PCR W (Wang et al., 2002) was designed to identify subspecies *fetus* by using *sapB2* as target. All Cfv strains are serotype A (with *sapA* genes) while Cff strains can be either serotype A or B (with either *sapA* or *sapB*). The reverse primer of this PCR is positioned in a variable region in the *sapB2* gene that remains highly homologous with *sapA2* (Willoughby et al., 2005), resulting in incorrect subspecies *fetus* identification. In the present study, PCR W showed low specificity which supported that this target is not suitable for identification of *C. fetus* subspecies.

One should realize that the sensitivity and specificity of the assays have been calculated for the strain set used in this study. Evaluation with another set of strains may result into a different sensitivity and specificity of the evaluated assays.

A goal of this study was to improve *C. fetus* diagnostics. As real-time PCR is rapid, easy and less labor-intensive than traditional PCR assays, it is the preferred PCR format for routine diagnostics. Two PCR assays, PCR A and PCR H, were able to identify *C. fetus* strains correctly at species level. Since the subspecies identification of PCR A showed the highest sensitivity (97%) and specificity (100%), the targets of this PCR were used to develop a real-time PCR assay, targeting gene *nahE* for species identification and insertion sequence IS*Cfe1* for subspecies *venerealis* identification.

Development of a real-time PCR assay for subspecies *venerealis* identification failed, as it was not possible to develop primers and probe on target gene IS*Cfe1* with 100% sensitivity and 100% specificity. The *C. fetus* species identification of the newly developed real-time PCR (*nahE*) assay performed excellently with 100% sensitivity and 100% specificity, with both purified DNA and boiled cell lysates, resulting in a reliable and rapid assay for *C. fetus* species identification. This real-time PCR (*nahE*) assay can be used to identify the species *fetus* when a *Campylobacter* strain is isolated. As obtaining isolates from samples still requires significant effort, the use of the new real-time PCR (*nahE*) assay directly on DNA material from clinical samples (e.g. boiled) would be the next step to improve *C. fetus* diagnostics. Research towards the development of new PCR assays based on recent genome sequences is needed in order to improve molecular diagnostic tools (e.g. real-time PCR) for identification of *C. fetus* subspecies.

5. Conclusion

Two PCR assays were able to correctly identify all *C. fetus* strains. The best performing PCR assay was converted into a real-time PCR assay, targeting gene *nahE* and insertion sequence IS*Cfe1*. The *C. fetus* species specific real-time PCR (*nahE*) assay showed 100%

sensitivity and 100% specificity, but the development of a subspecies *venerealis* specific real-time PCR (IS*Cfe1*) failed due to sequence variation of the target insertion sequence and prevalence in other *Campylobacter* species. None of the PCR assays tested were able to identify *C. fetus* strains correctly at subspecies level. Of the described methods, molecular analysis by AFLP or MLST is the most effective method for identification of *C. fetus* isolates at subspecies level; a persistent need for an easy, rapid and reliable molecular assay for subspecies identification remains.

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References

Abril, C., Vilei, E.M., Brodard, I., Burnens, A., Frey, J., Miserez, R., 2007. Discovery of insertion element IS*Cfe1*: a new tool for *Campylobacter fetus* subspecies differentiation. Clin. Microbiol. Infect. 13, 993–1000.

Abril, C., Brodard, I., Perreten, V., 2010. Two novel antibiotic resistance genes, Tet(44) and Ant(6)-Ib, are located within a transferable pathogenicity island in *Campylobacter fetus* subsp. *fetus*. Antimicrob. Agents Chemother. 54, 3052–3055.

Asakura, M., Samosornsuk, W., Taguchi, M., Kobayashi, K., Misawa, N., Kusumoto, M., Nishimura, K., Matsuhisa, A., Yamasaki, S., 2007. Comparative analysis of cytolethal distending toxin (*cdt*) genes among *Campylobacter jejuni*, *C. coli* and *C. fetus* strains. Microb. Pathog. 42, 174–183.

Bastyns, K., Chapelle, S., Vandamme, P., Goossens, H., de Wachter, R., 1994. Species-specific detection of campylobacters important in veterinary medicine by PCR amplification of 23S rDNA areas. Syst. Appl. Microbiol. 17, 563–568.

Blom, K., Patton, C.M., Nicholson, M.A., Swaminathan, B., 1995. Identification of *Campylobacter fetus* by PCR-DNA probe method. J. Clin. Microbiol. 33, 1360–1362.

Cardarelli-Leite, P., Blom, K., Patton, C.M., Nicholson, M.A., Steigerwalt, A.G., Hunter, S.B., Brenner, D.J., Barrett, T.J., Swaminathan, B., 1996. Rapid identification of *Campylobacter* species by restriction fragment length polymorphism analysis of a PCR-amplified fragment of the gene coding for 16S rRNA. J. Clin. Microbiol. 34, 62–67.

Casademont, I., Bizet, C., Chevrier, D., Guesdon, J.L., 2000. Rapid detection of *Campylobacter fetus* by polymerase chain reaction combined with non-radioactive hybridization using an oligonucleotide covalently bound to microwells. Mol. Cell. Probes 14, 233–240.

Chaban, B., Musil, K.M., Himsworth, C.G., Hill, J.E., 2009. Development of *cpn60*-based real-time quantitative PCR assays for the detection of 14 *Campylobacter* species and application to screening of canine fecal samples. Appl. Environ. Microbiol. 75, 3055–3061.

Chaban, B., Chu, S., Hendrick, S., Waldner, C., Hill, J.E., 2012. Evaluation of a *Campylobacter fetus* subspecies *venerealis* real-time quantitative polymerase chain reaction for direct analysis of bovine preputial samples. Can. J. Vet. Res. 76, 166–173.

Dekeyser, J., 1984. Campylobacter Infections in Man and Animals. In: Butzler, J.P. (Ed.), CRC Press Inc., Boca Raton, Florida.

Dohoo, I., Martin, W., Stryhn, H., 2003. Veterinary Epidemiologic Research. University of Prince Edward Island, Charlottetown, Canada, Atlantic Veterinary College.

Eaglesome, M.D., Sampath, M.I., Garcia, M.M., 1995. A detection assay for *Campylobacter fetus* in bovine semen by restriction analysis of PCR amplified DNA. Vet. Res. Commun. 19, 253–263.

Garcia, M.M., Eaglesome, M.D., Rigby, C., 1983. Campylobacters important in veterinary medicine. Vet. Bull. 53, 793–818.

Gorkiewicz, G., Feierl, G., Schober, C., Dieber, F., Kofer, J., Zechner, R., Zechner, E.L., 2003. Species-specific identification of campylobacters by partial 16S rRNA gene sequencing. J. Clin. Microbiol. 41, 2537–2546.

Gorkiewicz, G., Kienesberger, S., Schober, C., Scheicher, S.R., Gully, C., Zechner, R., Zechner, E.L., 2010. A genomic island defines subspecies-specific virulence features of the host-adapted pathogen *Campylobacter fetus* subsp. *venerealis*. J. Bacteriol. 192, 502–517.

Hum, S., Quinn, K., Brunner, J., On, S.L., 1997. Evaluation of a PCR assay for identification and differentiation of *Campylobacter fetus* subspecies. Aust. Vet. J. 75, 827–831.

Inglis, G.D., Kalischuk, L.D., 2003. Use of PCR for direct detection of *Campylobacter* species in bovine feces. Appl. Environ. Microbiol. 69, 3435–3447.

Iraola, G., Hernandez, M., Calleros, L., Paolicchi, F., Silveyra, S., Velilla, A., Carretto, L., Rodriguez, E., Perez, R., 2012. Application of a multiplex PCR assay for *Campylobacter fetus* detection and subspecies differentiation in uncultured samples of aborted bovine fetuses. J. Vet. Sci. 13, 371–376.

McMillen, L., Fordyce, G., Doogan, V.J., Lew, A.E., 2006. Comparison of culture and a novel 5′ Taq nuclease assay for direct detection of *Campylobacter fetus* subsp. *venerealis* in clinical specimens from cattle. J. Clin. Microbiol. 44, 938–945.

Moolhuijzen, P.M., Lew-Tabor, A.E., Wlodek, B.M., Aguero, F.G., Comerci, D.J., Ugalde, R.A., Sanchez, D.O., Appels, R., Bellgard, M., 2009. Genomic analysis of *Campylobacter fetus* subspecies: identification of candidate virulence determinants and diagnostic assay targets. BMC Microbiol. 9, 86.

Muller, W., Hotzel, H., Schulze, F., 2003. Identification and differentiation of *Campylobacter fetus* subspecies by PCR. Dtsch. Tierarztl. Wochenschr. 110, 55–59.

OIE, 2013a. http://www.oie.int/wahis_2/public/wahid.php/Wahidhome/Home Accession date: 23/04/2013.

OIE, 2013b. http://www.oie.int/international-standard-setting/terrestrial-code Accession date: 23/04/2013.

Oyarzabal, O.A., Wesley, I.V., Harmon, K.M., Schroeder-Tucker, L., Barbaree, J.M., Lauerman, L.H., Backert, S., Conner, D.E., 1997. Specific identification of *Campylobacter fetus* by PCR targeting variable regions of the 16S rDNA. Vet. Microbiol. 58, 61–71.

Salama, S.M., Garcia, M.M., Taylor, D.E., 1992. Differentiation of the subspecies of *Campylobacter fetus* by genomic sizing. Int. J. Syst. Bacteriol. 42, 446–450.

Schmidt, T., Venter, E.H., Picard, J.A., 2010. Evaluation of PCR assays for the detection of *Campylobacter fetus* in bovine preputial scrapings and the identification of subspecies in South African field isolates. J. S. Afr. Vet. Assoc. 81, 87–92.

Schulze, F., Bagon, A., Muller, W., Hotzel, H., 2006. Identification of *Campylobacter fetus* subspecies by phenotypic differentiation and PCR. J. Clin. Microbiol. 44, 2019–2024.

Spence, R.P., Bruce, I.R., McFadden, A.M., Hill, F.I., Tisdall, D., Humphrey, S., van der Graaf, L., van Bergen, M.A., Wagenaar, J.A., 2011. Cross-reaction of a *Campylobacter fetus* subspecies *venerealis* real-time PCR. Vet. Rec. 168, 131.

Tu, Z.C., Eisner, W., Kreiswirth, B.N., Blaser, M.J., 2005. Genetic divergence of *Campylobacter fetus* strains of mammal and reptile origins. J. Clin. Microbiol. 43, 3334–3340.

van Bergen, M.A.P., Dingle, K.E., Maiden, M.C., Newell, D.G., van der Graaf-van Bloois, L., van Putten, J.P., Wagenaar, J.A., 2005a. Clonal nature of *Campylobacter fetus* as defined by multilocus sequence typing. J. Clin. Microbiol. 43, 5888–5898.

van Bergen, M.A.P., Simons, G., van der Graaf-van Bloois, L., van Putten, J.P., Rombout, J., Wesley, I., Wagenaar, J.A., 2005b. Amplified fragment length polymorphism based identification of genetic markers and novel PCR assay for differentiation of *Campylobacter fetus* subspecies. J. Med. Microbiol. 54, 1217–1224.

Vargas, A.C., Costa, M.M., Vainstein, M.H., Kreutz, L.C., Neves, J.P., 2003. Phenotypic and molecular characterization of bovine *Campylobacter fetus* strains isolated in Brazil. Vet. Microbiol. 93, 121–132.

Véron, M., Chatelain, R., 1973. Taxonomy study of the genus *Campylobacter* Sebald and Verón and designation of the neotype strain for the type species, *Campylobacter fetus* (Smith and Taylor) Sebald and Verón. Int. J. Syst. Bacteriol. 23, 122–134.

Wagenaar, J.A., van Bergen, M.A.P., Newell, D.G., Grogono-Thomas, R., Duim, B., 2001. Comparative study using amplified fragment length polymorphism fingerprinting, PCR genotyping, and phenotyping to differentiate *Campylobacter fetus* strains isolated from animals. J. Clin. Microbiol. 39, 2283–2286.

Wang, G., Clark, C.G., Taylor, T.M., Pucknell, C., Barton, C., Price, L., Woodward, D.L., Rodgers, F.G., 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.

Wesley, I.V., Wesley, R.D., Cardella, M., Dewhirst, F.E., Paster, B.J., 1991. Oligodeoxynucleotide probes for *Campylobacter fetus* and *Campylobacter hyointestinalis* based on 16S rRNA sequences. J. Clin. Microbiol. 29, 1812–1817.

Willoughby, K., Nettleton, P.F., Quirie, M., Maley, M.A., Foster, G., Toszeghy, M., Newell, D.G., 2005. A multiplex polymerase chain reaction to detect and differentiate *Campylobacter fetus* subspecies *fetus* and *Campylobacter fetus* subspecies *venerealis*: use on UK isolates of *C. fetus* and other *Campylobacter* spp J. Appl. Microbiol. 99, 758–766.

Yamazaki, W., Taguchi, M., Ishibashi, M., Nukina, M., Misawa, N., Inoue, K., 2009. Development of a loop-mediated isothermal amplification assay for sensitive and rapid detection of *Campylobacter fetus*. Vet. Microbiol. 136, 393–396.

Yamazaki, W., Taguchi, M., Misawa, N., 2010. Development of loop-mediated isothermal amplification and PCR assays for rapid and simple detection of *Campylobacter fetus* subsp. *venerealis*. Microbiol. Immunol. 54, 398–404.