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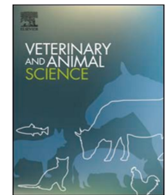

Accurate and fast identification of *Campylobacter fetus* in bulls by real-time PCR targeting a *16S rRNA* gene sequence

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ABSTRACT

Campylobacter fetus is an important animal pathogen that causes infectious infertility, embryonic mortality and abortions in cattle and sheep flocks. There are two recognized subspecies related with reproductive disorders in livestock: *Campylobacter fetus* subsp. *fetus* (Cff) and *Campylobacter fetus* subsp. *venerealis* (Cfv). Rapid and reliable detection of this pathogenic species in bulls is of upmost importance for disease control in dairy and beef herds as they are asymptomatic carriers. The aim of the present work was to assess the performance a real-time PCR (qPCR) method for the diagnosis of *Campylobacter fetus* in samples from bulls, comparing it with culture and isolation methods. 520 preputial samples were both cultured in Skirrow's medium and analyzed by qPCR. The estimated sensitivity of qPCR was 90.9% (95% CI, 69.4%–100%), and the specificity was 99.4% (95% CI, 98.6% - 100%). The proportion of *C. fetus* positive individuals was 2.1% by isolation and 2.5% by qPCR. Isolates were identified by biochemical tests as Cfv ($n = 9$) and Cff ($n = 2$). Our findings support the use of qPCR for fast and accurate detection of *C. fetus* directly from field samples of preputial smegma of bulls. The qPCR method showed to be suitable for massive screenings because it can be performed in pooled samples without losing accuracy and sensitivity.

1. Introduction

Campylobacter genus is composed by Gram-negative epsilon-proteobacteria specially adapted to vertebrate hosts. Some species are pathogens of livestock and also have reservoirs in wild and domestic fauna. The species *Campylobacter fetus* is an important animal pathogen that is a primary cause of infertility, embryonic mortality and sporadic abortions in bovines and ovines. This species is currently divided into the subspecies *Campylobacter fetus* subsp. *fetus* (Cff), *Campylobacter fetus* subsp. *venerealis* (Cfv) and *Campylobacter fetus* subsp. *testudinum* (Cft) (Fitzgerald et al., 2014; Veron & Chatelain, 1973). While Cfv is host-restricted and isolated almost exclusively from the bovine genital tract, Cff has been isolated from many hosts including sheep, cattle and humans (Iraola et al., 2017; Wagenaar et al., 2014). Cft has been more recently proposed based on the characterization of genetically divergent strains isolated from reptiles and sick humans (Dingle et al., 2010;

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Fitzgerald et al., 2014; Patrick et al., 2013).

Bovine Genital Campylobacteriosis (BGC) is a venereal disease associated with lowered pregnancy rates and sporadic abortion (Bryner, 1964). Campylobacteriosis has great economic impact and is a serious concern for cattle industry worldwide (On, 2001). Infected animals have trade restrictions and therefore it is mandatory to report outbreaks to the World Organization for Animal Health (OIE). BGC has been traditionally associated to Cfv, but the Cff subspecies also occurred in cows with reproductive disorders and is found in preputial cavities, vaginal mucus and in the organs of aborted fetuses. As Cfv and Cff are not clearly differentiated at the genomic level (Calleros et al., 2016; van der Graaf-van Bloois et al., 2014), the presence of any of them is usually considered evidence of a reproductive disease (Repisoet al., 2005).

In Uruguay, cattle pregnancy rate has a mean of 73% and the reproductive losses between the diagnosis of gestation and weaning have been estimated to be 10.3% (Uruguayan Ministry of Agriculture, 2018). The total amount of reproductive losses is usually accepted to be multifactorial including genetic incompatibilities, nutritional and toxicological aspects and infectious agents which interact with one another depending on the type of herd and system. This challenges the correct diagnosis of losses. Infectious diseases are the main cause of approximately 50% of reproductive failures (Campero et al., 2005). BGC was reported for the first time in Uruguay in 1967 with a wide distribution in dairy cattle in the south of the country (Errico, De Freitas, Tedesco & Barriola, 1976). *Campylobacter fetus* was present in 2.6% of the bulls in 2005 (Repisoet al., 2005) and it was also identified as the possible cause of abortion in 16% of 431 aborted fetuses examined in 2006 (Easton, 2006). The species *C. fetus* was associated with 2% of a series of 102 abortions analyzed during 2018 (Macías-Rioseco, 2018). The frequency of campylobacteriosis as a cause of infertility in cows and its influence in the pregnancy and weaning rates is still unclear, but the disease is frequently diagnosed in our country by public and private laboratories using different techniques, including culture and isolation, end point PCR and immunofluorescence. Accordingly, epidemiological surveillance of this disease is essential, as it may have an important role in the observed low pregnancy and weaning rates in Uruguay. Detection of carrier bulls is particularly required in order to control campylobacteriosis. Bulls are the main spreading source of the pathogen within a herd during breeding. The continuous sampling of bulls is essential to reduce transmission, thus improving conception rates in heifers and shortening service periods. Preputial sampling and diagnosis of bulls before the service is a widely spread control measure carried out by field vets in our productive systems. Nevertheless, there are still productive systems that lack veterinary assistance. It is essential that sold bulls are certified free of the disease.

The diagnosis of BGC in cases of infertility is difficult, and there has been a continuous search for practical, fast and low-cost methodologies. The combination of culture isolation and biochemical tests is useful to analyze different kinds of samples, even those with a low bacterial count (like preputial washings from bulls). This methodology is considered the gold standard for the diagnosis and identification of *C. fetus* and its subspecies (OIE, 2008) and is well standardized and extensively used. However, it is time-consuming and labor intensive, a disadvantage when processing samples at a large-scale. This method presents additional difficulties, such as the interference with other microorganisms because samples come from highly contaminated environments (Bolton, Holt & Hutchinson, 1984; Lander, 1990), and the microaerophilic conditions required for its growth. These fastidious growth requirements motivated the development of alternative diagnostic methods.

Molecular methods using PCR have become a suitable alternative for fast and highly specific bacterial diagnosis. Several studies have proposed methods for identifying the species *C. fetus* and its subspecies using end point PCRs (Abril et al., 2007; Linton, Owen & Stanley, 1996; Schouls et al., 2003; Schulze, Bagon, Müller & Hotzel, 2006; Van Bergen, Linnane, van Putten & Wagenaar, 2005; Vargas, Costa, Vainstein, Kreutz & Neves, 2003). The most widely used assay is a multiplex PCR that detects the species *C. fetus* and differentiates between its subspecies (Hum, Quinn, Brunner & On, 1997). More recently, another multiplex PCR assay was proposed, based on the *virB11* gene which is present in a pathogenic island of Cfv (Gorkiewicz et al., 2010; Iraola et al., 2012). These methods are accurate to identify *C. fetus* species. Nevertheless, there are several evidences that there isn't a high concordance between molecular assays and biochemical tests for subspecies differentiation (Calleros et al., 2016). To the best of our knowledge, there is no molecular method whose results correspond to that of the biochemical tests, which is the gold standard for subspecies differentiation.

Real-time PCR methods have several advantages over the end point PCR methods, such as the high sensitivity and specificity, speed and reduced risk of cross-contamination. Because of the advantages of the 16S *rRNA* genes for reliable detection of bacteria (Blom, Patton, Nicholson & Swaminathan, 1995; Linton et al., 1996; Weisburg, Barns, Pelletier & Lane, 1991) a qPCR assay targeting *rRNA* gene sequences for the specific detection of *C. fetus* was developed (Iraola et al., 2016). However, the clinical application of this qPCR test has not been yet established in routine surveillance.

The primary goal of this study was to assess the clinical usefulness of this qPCR for the diagnosis of *C. fetus* in preputial scrapings. The method is compared with the standard bacteriological method to determine the clinical sensitivity and specificity of the technique for preputial smegma samples.

2. Materials and methods

2.1. Samples

A total of 520 samples were obtained from bull preputial smegma. Samples were collected from two slaughterhouses with batches from 98 different farms. The farm location of the sampled bulls in this study was distributed in 17 out of 19 political divisions of the country (*Departamentos*). Samples were collected between August 2017 and July 2019 in 13 independent samplings. To obtain samples, the external part of the penis and prepuce was recovered during the slaughter and scraped with a disposable spatula obtaining the preputial smegma with the least possible contamination. The material collected in the scraper or spatula was discharged in individual tubes with 3 mL sterile PBS and placed in an isothermal box at room temperature. The samples were immediately transported to the laboratory for culture and molecular analysis.

2.2. Culture and isolation

Samples were cultured within 4 h after collection. Briefly, 150 μl of each sample were inoculated in Agar Skirrow Medium and incubated at 37 °C in a microaerophilic atmosphere (CampyGen®, Oxoid, UK) with 5–10% oxygen, 5–10% carbon dioxide and 5–9% hydrogen for 48 h. Colonies were visually inspected by naked eye and under a phase contrast microscope. At least three suspected *C. fetus* colonies were reinoculated in Columbia blood agar medium (Oxoid, UK) in independent plates to obtain the isolates. Subspecies classification was phenotypically determined with the 1% glycine tolerance test and the hydrogen sulfide (H_2S) production test in a medium with Triple Sugar Iron (TSI). Isolates were frozen in *Brucella* broth with 16% glycerol in liquid nitrogen for conservation.

2.3. Molecular assays

Molecular assays and culture methods were performed simultaneously. DNA extraction was performed individually from 500 μL of each sample using a modified fast boiling method (Schunck, Kraft & Truyen, 1995).

An end point PCR control method was used to assess the quality of the extracted DNA. This method amplifies a fragment of the *16S rRNA* gene using a new combination of previously described universal primers

(Delong, 1992; Jiang et al., 2006; Lane, 1985; Weisburg et al., 1991). For this assay, PCR was carried out in a mix containing 0.5 mM of each dNTP, 1 × reaction buffer, 2.5 mM $MgCl_2$, 1.0 μM Bac27F/534R primer set, and 1.5 U Taq DNA polymerase (Thermo Scientific, USA) in a final volume of 20 μL. The following cycling conditions were used: an initial denaturation for 3 min at 94 °C followed by 30 cycles of denaturation for 30 sec at 94 °C, annealing for 45 sec at 54 °C, and extension for 1:30 min at 72 °C. Amplicons were separated in 1.0% agarose gels and stained with ethidium bromide. This control method was applied to all samples individually, and also pooled in groups of three and five.

Real-time PCR was carried out in duplicates following a previously described protocol (Iraola et al., 2016). The reaction was carried out in 25μL containing 1 × HotRox Master Mix (Bioron), 8 mM $MgCl_2$, 0.3 μM of each primer, 0.1 μM TaqMan-MGB probe (Applied Biosystems) and 1 to 5 μL of genomic DNA.

Thermocycling was performed on an ABIPrism 7500 analyzer (Applied Biosystems, FC, USA).

Samples were tested individually and in pools of three or five. Pools were made using equal volumes of DNA extraction from each sample. The amount of template DNA for the PCR and qPCR reactions was modified according to the pooling scheme.

The performance of the individual samples and pools were compared using CT values and final fluorescence values. The lower limit of the linear dynamic range of the assay was established in 10^2 genome copies per reaction, with a mean CT value of 37 (Iraola et al., 2016). Therefore, samples with a CT minor to 37 were considered positive. When positive pools were identified, the qPCR assay was repeated individually to detect the positive samples.

Species identification of the isolates was confirmed by amplification and sequencing of a fragment of the *16S rRNA* gene with primers C412F and C1288F using previously described conditions (Linton et al., 1996). Additional identification of *C. fetus* and differentiation of Cff and Cfv was performed for each isolate by two previously described multiplex PCR methods (Hum et al., 1997; Iraola et al., 2012).

For real-time and end point PCRs, positive and negative controls were included in every assay. Two positive controls were included as DNA extractions from a pure *C. fetus* culture and from a positive sample. Two negative controls were used: DNA extraction from a negative sample and nuclease free water.

2.4. Statistical analysis

Sensitivity and specificity of qPCR and the *kappa* value (Thrusfield, 1995) to measure the agreement between the culture and the qPCR techniques were calculated using the Epidat 3.1 software.

The proportion of positive cases was determined by the Wilson score method (Newcombe, 1998), which provided the estimated mean and the ranges between the lower limit and the upper limit expressed as a percentage with a confidence interval of 95%. It was considered that there was evidence of statistical differences in the case that no values overlapped in the comparison between the ranges of the lower limit and the upper limit.

3. Results

3.1. Culture and isolation

A total of 520 samples of preputial smegma were cultured in a selective medium. Eleven samples coming from 8 different farms resulted in the isolation of *C. fetus*. Isolates were identified by biochemical tests as Cfv ($n = 9$) and Cff ($n = 2$) (Table 1).

3.2. Molecular assays

The DNA quality control method used, which amplifies a fragment of the *16S rRNA* gene was the first step during the analysis of pools and samples. All samples and pools showed a positive result.

There were no differences between pools and individual samples in presence/absence results in qPCR assays. CT values ranged from 26 to 36 in samples and in pools. CT values of pools (3 and 5 samples each) and individual positive samples from each pool were concordant. Due to this, samples were analyzed in pools of five. In all cases where positive pools were identified, the qPCR assay was performed individually and detected at least one positive sample. No differences between the duplicates were found neither in the samples nor in the controls.

According to qPCR, thirteen samples were positive which included 10 with isolation of *C. fetus* and 3 samples where the isolation was unsuccessful (Table 2).

Comparison of sequences from a fragment of the *16S rRNA* gene (Linton et al., 1996) with those on the NCBI nucleotide database showed 99.7% to 100% identity to several published *C. fetus* sequences, identifying the isolates as *C. fetus*. Biochemical and molecular assays confirmed this result. All isolates were identified by both molecular assays as Cfv (Table 1).

Table 1
Phenotypic testing and genotypic characterization (multiplex PCR) of the isolates obtained.

Isolate	Phenotypic typing[1]	MultiplexPCR A[2]	MultiplexPCR B[3]
Uy/R18	Cfv	Cfv	Cfv
Uy/V28	Cfv	Cfv	Cfv
Uy/W8	Cfv	Cfv	Cfv
Uy/W11	Cfv	Cfv	Cfv
Uy/X29	Cfv	Cfv	Cfv
Uy/AD29	Cfv	Cfv	Cfv
Uy/AE9	Cff	Cfv	Cfv
Uy/AE14	Cfv	Cfv	Cfv
Uy/AE16	Cfv	Cfv	Cfv
Uy/AF47	Cfv	Cfv	Cfv
Uy/AF53	Cff	Cfv	Cfv

[1] Glycine tolerance and H_2S production. [2]As described in Hum et al., 1997. [3]As described in Iraola et al., 2012.

3.3. Statistical analysis

The proportion of *C. fetus* positive individuals determined by the Wilson score method was 2.1% (95% CI, 1.1% - 3.1%) by culture and isolation and 2.5% (95% CI, 1.5% - 3.8%) by qPCR. There was no evidence of statistical differences between the results of culture and qPCR.

For the estimation of the sensitivity and specificity of qPCR, the results from culture and isolation were taken as the reference, obtaining a sensitivity of 90.9% (95% CI, 69.4%–100%) and specificity of 99.4% (95% CI, 98.6% - 100%) (Table 2).

The *kappa* value obtained when comparing the diagnostic techniques was 0.8, with a standard error of 0.08 and a confidence interval of 95%.

4. Discussion

The qPCR technique used in this research was previously developed by our group and targets a unique region of the *16S rRNA* gene of *C. fetus*. It has sensitivity and specificity of 100% obtained using isolated strains of *C. fetus*, with no cross-reaction with isolates from other

Table 2
Number of positive animals by culture and qPCR. Sensitivity and specificity of the qPCR assay, calculated using culture as the reference technique. 95% confidence intervals (CI) were calculated.

		Culture		
		Positive	**Negative**	**Sensitivity (CI)**
qPCR	Positive	10	3	90.9% (69.4–100)
	Negative	1	506	
Specificity (CI)		99.4% (98.7–100)		

bacterial species (Iraola et al., 2016). The aim of the present study was to compare the performance of this assay to culture and isolation, the current gold standard for *C. fetus* detection. We tested 520 samples of preputial scrapings, and obtained 11 isolates that were confirmed to be *C. fetus* by three separated molecular assays: sequencing of a fragment of the *16S rRNA* gene (Linton et al., 1996) and two end point PCRs that detect the specific gene *cstA* (Table 1) (Hum et al., 1997; Iraola et al., 2012).

Here, an end point PCR DNA control was standardized using universal primers that detect every bacterial species. The result of this control was positive in all samples, suggesting good quality of the extracted DNA. As the theoretic sensitivity of end point PCR is inferior to that of qPCR, we consider this result is satisfactory enough to establish that the extraction method chosen for the preputial samples is adequate.

Our results showed that the qPCR used in this research has a sensitivity and specificity of 91% and 99%, respectively, in relation to the gold standard culture and isolation technique in samples of preputial smegma. The high sensitivity and specificity of this qPCR suggests that the 3 samples that tested positive for qPCR and were negative in the culture were true positives for qPCR and false negatives in the cultures. The sensitivity of the culture reported in previous studies is between 25% and 90% (Guerra, Chaban, Hill, Waldner & Hendrick, 2014), reinforcing this hypothesis and providing additional relevance to our results.

As a *kappa* value equal to or greater than 0.40 is acceptable and considered excellent if it is higher than 0.75 (Thrusfield, 1995), the *kappa* value of 0.8 between the culture and the qPCR indicates an excellent concordance between both methods.

Our findings confirm that in Uruguay, *C. fetus* is still present in preputial smegma samples from slaughterhouse bulls. The proportion of *C. fetus*-infected animals (2.1%) is similar to the value of 2.6% reported in 2005 (Repisoet al., 2005) using the culture and isolation method.

Subspecies differentiation is still a matter of discussion in the scientific community. Our results confirm the previously reported discrepancies between biochemical and molecular assays (Calleros et al., 2016) (Table 1), mostly due to horizontal transfer of genetic material (Abdel-Glil, Hotzel, Tomaso & Linde, 2020; Silva et al., 2020). This reinforces the idea that there is still necessary to investigate further on this issue. Regardless of that issue, we found that Cfv is the predominant subspecies circulating in bovines in Uruguay, as previously reported (Repisoet al., 2005). Since Cff can also be present in the bull's prepuce, our results confirm the usefulness of a method that is suitable to detect any variant of *C. fetus*, including both mammal-associated subspecies.

The protocol used here is a sensitive, specific, reproducible and less time consuming technique (Iraola et al., 2016), which makes it a good option as a screening technique for the detection of *C. fetus* in clinical samples from bulls. Additionally, the greater sensitivity of the qPCR in relation to the end point PCR allows the analysis of pools of five samples which, together with the use of a rapid and low cost DNA extraction method, significantly decreases the cost and time of processing when dealing with a great number of samples.

To ensure the feasibility of the method for use in live animals, we performed a preliminary study using prepuce scrapings from 168 bulls and the same methodology as in slaughterhouse bulls. Results showed a sensitivity and specificity of 100% and 99%, respectively (Supplementary material S1). Even when these results have to be confirmed using a larger amount of samples, we consider they are very promising, as they are equivalent to those presented here using slaughterhouse bulls.

An advantage of PCR and molecular biology techniques is that they are based on DNA detection and therefore can detect the agent in low loads. In a disease such as BGC, where the number of viable bacteria obtained in the sample of preputial smegma cannot be predicted, and the transport conditions strongly affect the culture outcome, appropriate identification of the microorganism is difficult. Thus, relying on a PCR technique is an advantage for diagnosis.

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6. Ethical statement

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. The ethics committee (CEUA Facultad de Ciencias), in their resolution from 06/30/2015, certified that protocol revision was not necessary for being a routinely veterinary procedure.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.vas.2020.100163.

References

Abdel-Glil, M. Y., Hotzel, H., Tomaso, H., & Linde, J. (2020). Phylogenomic analysis of Campylobacter fetus reveals a clonal structure of insertion element ISCfe1 positive genomes. *Frontiers in Microbiology, 11*. https://doi.org/10.3389/fmicb.2020.585374 (November).

Abril, C., Vilei, E. M., Brodard, I., Burnens, A., Frey, J., & Miserez, R. (2007). Discovery of insertion element ISCfe1: a new tool for Campylobacter fetus subspecies differentiation. *Clinical Microbiology and Infection : The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases, 13*(10), 993–1000, 10.1111/j.1469-0691.2007.01787.x.

Blom, K., Patton, C. M., Nicholson, M., & Swaminathan, B. (1995). Identification of Campylobacter fetus by PCR-DNA probe method. *J Clinical Microbiology, 33*(5), 1360–1362, 10.1128/JCM.33.5.1360-1362.1995.

Bolton, F. J., Holt, A. V., & Hutchinson, D. N. (1984). Campylobacter biotyping scheme of epidemiological value. *J Clinical Pathology, 37*(6), 677–681. https://doi.org/10.1136/jcp.37.6.677.

Bryner, J. H. (1964). Vibrio infection of the digestive organs of cattle. *American J Veterinary Research, 25*, 1048–1050.

Calleros, L., Betancor, L., Iraola, G., Méndez, A., Morsella, C., Paolicchi, F., et al. (2016). Assessing the intra-species genetic variability in the clonal pathogen Campylobacter fetus: CRISPRs are highly polymorphic DNA markers. *J Microbiological Methods, 132*, 86–94. https://doi.org/10.1016/j.mimet.2016.11.012.

Campero, C.M., .Anderson, M.L., .Walker, R.L., .Blanchard, P.C., .Barbano, L., Chiu, P. et al. (2005). Immunohistochemical identification of Campylobacter fetus in natural cases of bovine and ovine abortions. Journal of Veterinary Medicine. B, Infectious Diseases and Veterinary Public Health, 52(3), 138–141. 10.1111/j.1439-0450.2005.00834.x.

Delong, E. F. (1992). Archea in coastal marine enviroment. *Proceedings of the National Academy of Sciences of the United States of America, 89*, 5685–5689, 10.1073/pnas.89.12.5685.

Dingle, K. E., Blaser, M. J., Tu, Z.-C. C., Pruckler, J., Fitzgerald, C., van Bergen, M. A. P. P., et al. (2010). Genetic relationships among reptilian and mammalian Campylobacter fetus strains determined by multilocus sequence typing. *J Clinical Microbiology, 48*(3), 977–980, 10.1128/JCM.01439-09.

Easton, M. (2006). *Estudio patológico de las principales causas infecciosas en el aborto bovino en uruguay. (Spanish). master thesis* (pp. 1–60). UdelaR, Uruguay: Maestría En Salud Animal.

Errico, F., De Freitas, A., Tedesco, L., & Barriola, J. (1976). Tipificación, distribución y síndrome asociado con el aislamiento de cepas Campylobacter fetus de bovinos del Uruguay. (Spanish). *Gaceta Veterinaria, 39*, 232–237.

Fitzgerald, C., Tu, Z. C., Patrick, M., Stiles, T., Lawson, A. J., Santovenia, M., et al. (2014). Description of Campylobacter fetus subsp. testudinum subsp. nov., isolated

from humans and reptiles. *Int J Systematic and Evolutionary Microbiology*, 1–16. https://doi.org/10.1099/ijs.0.057778-0.

Gorkiewicz, G., Kienesberger, S., Schober, C., Scheicher, S. R., Gülly, C., Zechner, R., et al. (2010). A genomic island defines subspecies-specific virulence features of the host adapted pathogen Campylobacter fetus subsp. *Venerealis*, 10.1128/JB.00803-09.

Guerra, A. G., Chaban, B., Hill, J. E., Waldner, C. L., & Hendrick, S. H. (2014). Clinical sensitivity and specificity of a real-time PCR assay for Campylobacter fetus subsp venerealis in preputial samples from bulls. *American J Veterinary Research, 75*(9), 851–860, 10.2460/ajvr.75.9.851.

Hum, S., Quinn, K., Brunner, J., & On, S. L. W. (1997). Evaluation of a PCR assay for identification and differentiation of Campylobacter fetus subspecies. *Aust Vet J, 75* (11), 827–831. https://doi.org/10.1111/j.1751-0813.1997.tb15665.x.

Iraola, G., Forster, S. C., Kumar, N., Lehours, P., Bekal, S., García-Peña, F. J., & L. awley, T. D. (2017). Distinct Campylobacter fetus lineages adapted as livestock pathogens and human pathobionts in the intestinal microbiota. *Nature Communications, 8*(1), 1367, 10.1038/s41467-017-01449-9.

Iraola, G., Hernández, M., Calleros, L., Paolicchi, F., Silveyra, S., Velilla, A., et al. (2012). Application of a multiplex PCR assay for Campylobacter fetus detection and subspecies differentiation in uncultured samples of aborted bovine fetuses. *J Veterinary Science, 13*(4), 371–376. https://doi.org/10.4142/jvs.2012.13.4.371.

Iraola, G., Pérez, R., Betancor, L., Marandino, A., Morsella, C., Méndez, A., et al. (2016). A novel real-time PCR assay for quantitative detection of Campylobacter fetus based on ribosomal sequences. *BMC Veterinary Research, 12*(1), 286, 10.1186/s12917-016-0913-3.

Jiang, H., Dong, H., Zhang, G., Yu, B., Chapman, L. R., & Fields, M. W. (2006). Microbial diversity in water and sediment of Lake Chaka, an athalassohaline lake in northwestern China. *Applied and Environmental Microbiology, 72*(6), 3832–3845, 10.1128/AEM.02869-05.

Lander, K. P. (1990). The development of a transport and enrichment medium for Campylobacter fetus. *British Veterinary J, 146*(4), 327–333, 10.1016/S0007-1935(11)80025-4.

Lane, D. J. (1985). Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. *Proc. Natl Acad. Sci. USA, 82*, 6955–6959. https://doi.org/10.1073/pnas.82.20.6955.

Linton, D., Owen, R. J., & Stanley, J. (1996). Rapid identification by PCR of the genus Campylobacter and of five Campylobacter species enteropathogenic for man and animals. *Research in Microbiology, 147*(9), 707–718. https://doi.org/10.1016/S0923-2508(97)85118-2.

Macías-Rioseco, M. (2018). *Causas de aborto en bovinos lecheros de Uruguay. (Spanish). doctoral thesis* (pp. 1–79). UdelaR, Uruguay: Doctorado en Salud Animal.

Newcombe, R. G. (1998). Two-sided confidence intervals for the single proportion: Comparison of seven methods. *Statistics in Medicine, 17*(8), 857–872, 10.1002/(SICI)1097-0258(19980430)17:8<857::AID−SIM777>3.0.CO;2-E.

OIE. (2008). OIE terrestrial manual, chapter 2.4.5. *Bovine Genital Campylobacteriosis*, 661–670.

On, S. L. W. (2001). Taxonomy of Campylobacter, Arcobacter, Helicobacter and related bacteria: current status, future prospects and immediate concerns. *J Applied Microbiology, 90*(S6), 1S–15S, 10.1046/j.1365-2672.2001.01349.x.

Patrick, M. E., Gilbert, M. J., Blaser, M. J., Tauxe, R. V., Wagenaar, J. A., & Fitzgerald, C. (2013). Human infections with new subspecies of Campylobacter fetus. *Emerging Infectious Diseases, 19*(10), 1678–1680, 10.3201/eid1910.130883.

Repiso, M. V., Gil, A., Bañales, P., D'Anatro, N., Fernandez, L., Guarino, H., et al. (2005). Prevalencia de las principales enfermedades infecciosas que afectan el comportamiento reproductivo en la ganadería de carne y caracterización de los establecimientos de cría del Uruguay. *(Spanish). Veterinaria, (Montevideo), 40*, 5–28.

Schouls, L. M., Reulen, S., Duim, B., Wagenaar, J. A., Willems, R. J. L., & Dingle, K. E. (2003). Comparative genotyping of Campylobacter jejuni by Amplified Fragment Length Polymorphism, Multilocus Sequence Typing, and Short Repeat Sequencing : strain Diversity, Host Range, and Recombination. *Society, 41*(1), 15–26. https://doi.org/10.1128/JCM.41.1.15.

Schulze, F., Bagon, A., Müller, W., & Hotzel, H. (2006). Identification of Campylobacter fetus subspecies by phenotypic differentiation and PCR. *J Clinical Microbiology, 44* (6), 2019–2024, 10.1128/JCM.02566-05.

Schunck, B., Kraft, W., & Truyen, U. (1995). A simple touch-down polymerase chain reaction for the detection of canine parvovirus and feline panleukopenia virus in feces. *J Virological Methods, 55*(3), 427–433, 10.1016/0166-0934(95)00069-3.

Silva, M. F., Duarte, A., Pereira, G., Mateus, L., Lopes-da-Costa, L., & Silva, E. (2020). Assessment of Campylobacter fetus subsp. venerealis molecular diagnosis using clinical samples of bulls. *BMC Veterinary Research, 16*(1), 1–9. https://doi.org/10.1186/s12917-020-02634-7.

Thrusfield, M. (1995). *Veterinary epidemiology*. Oxford, UK: Blackwell Science.

Uruguayan Ministry of Agriculture. (2018). Resultados de la encuesta Ganadera Nacional 2016. (Spanish). *MGAP, 47*(2), 85–94, 10.1016/j.jval.2013.08.1935.

Van Bergen, M. A. P., Linnane, S., van Putten, J. P. M., & Wagenaar, J. A. P. (2005). Global detection and identification of Campylobacter fetus subsp. venerealis. *Revue Scientifique et Technique (International Office of Epizootics), 24*(3), 1017–1026. http://www.ncbi.nlm.nih.gov/pubmed/16642772.

van der Graaf-van Bloois, L., Miller, W. G., Yee, E., Rijnsburger, M., Wagenaar, J. A., & Duim, B. (2014). Inconsistency of phenotypic and genomic characteristics of Campylobacter fetus subspecies requires re-evaluation of current diagnostics. *J Clinical Microbiology, 52*(September), 4183–4188, 10.1128/JCM.01837-14.

Vargas, A., 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. *Veterinary Microbiology, 93*(2), 121–132, 10.1016/S0378-1135(03)00018-X.

Veron, M., & Chatelain, R. (1973). Taxonomic Study of the Genus Campylobacter Sebald and Veron and Designation of the Neotype Strain for the Type Species, Campylobacter fetus (Smith and Taylor) Sebald and Veron. *Int J Systematic Bacteriology, 23*(2), 122–134, 10.1099/00207713-23-2-122.

Wagenaar, J. A., Van Bergen, M. A. P., Blaser, M. J., Tauxe, R. V., Newell, D. G., & Van Putten, J. P. M. (2014). Campylobacter fetus infections in humans: Exposure and disease Accepted. Clinical Infectious Diseases. *An Official Publication of the Infectious Diseases Society of America, 58*(11), 1–26. https://doi.org/10.1093/cid/ciu085.

Weisburg, W. G., Barns, S. M., Pelletier, D. A., & Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriology, 173*(2), 697–703, 10.1128/jb.173.2.697-703.1991.