Abanico Veterinario. January-December 2023; 13:1-10. http://dx.doi.org/10.21929/abavet2023.6
Case study. Received: 01/08/2022. Accepted:28/02/2023. Published: 28/04/2023. Code: e2022-57.
https://www.youtube.com/watch?v=DIZAWlMFh80

Frequency of *Campylobacter fetus* in bulls in the central zone of Tamaulipas Mexico

Frecuencia de *Campylobacter fetus* en bovinos sementales en la zona centro de Tamaulipas México

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ABSTRACT

Bovine Genital Campylobacteriosis (BGC) is a contagious infectious disease that affects cattle. The disease is considered obligatory reporting and it is included in list B of terrestrial animal diseases according to the World Organization for Animal Health (WOAH). In ruminants, *Campylobacter fetus* subsp. *fetus* (*Cff*) has been shown to affect the enteric system, especially the intestine, and it is one of the main causes of infertility and abortion in cattle, sheep and goats. It has been reported in several countries around the world. In Mexico, the presence of this pathogen has not been studied, so the objective of this research was to detect *Campylobacter fetus* subsp. *veneralis* (*CFv*) by the PCR technique, and to perform its genetic characterization in bulls from the central area of Tamaulipas, Mexico. This is the first report of this pathogen by molecular methods in this country.

Keywords: *Campylobacter fetus veneralis*, bulls, sperm, preputial lavage, Mexico.

RESUMEN

La Campilobacteriosis Genital Bovina (CGB) es una enfermedad infecto contagiosa que afecta al ganado bovino. La enfermedad es considerada de reporte obligatorio y está incluida en la lista B de enfermedades de los animales terrestres según la Organización Mundial de Salud Animal (OMSA). En rumiantes *Campylobacter fetus* subsp. *fetus* se ha demostrado que afecta el sistema entérico, especialmente el intestino y es una de las principales causas de infertilidad y aborto en bovinos, ovinos y caprinos. Se ha reportado en varios países del mundo. En México poco se ha estudiado la presencia de este patógeno; en este estudio se comprobó la existencia de *Campylobacter fetus* subsp. *veneralis* en toros de la zona centro de Tamaulipas México por la técnica de PCR y secuenciación del producto amplificado, por lo que representa el primer reporte de este patógeno por métodos moleculares en este país.

Palabras claves: *Campylobacter fetus veneralis*, toros, semen, lavado prepucial, México.

INTRODUCTION

Bovine genital campylobacteriosis (BGC) is an infectious disease affecting cattle. The *Campylobacter* genus is responsible for this disease. They are Gram-negative, spiral-shaped, motile bacteria. Currently, 32 *Campylobacter* species are recognized (Chukwu *et al.,* 2019*)* which *Campylobacter fetus subsp. fetus* (*Cff*) and *Campylobacter fetus* subsp. *venerealis* (*Cfv*) affect the reproductive system of cattle (Chiapparrone *et al.,* 2014). The disease is considered a mandatory reportable disease, included in List B of terrestrial animal diseases according to the World Organization for Animal Health (WOAH), formerly OIE (Tshipamba *et al*., 2020). In ruminants *Cff* has been shown that the pathogen affects the enteric system, especially the intestine (Li *et al*., 2022), on the other hand, bulls with *Cfv* are reservoir because the bacteria live and adapt in preputial cysts, while in cows colonizations are in vagina, cervix and uterus ((Cagnoli *et al.,* 2020 and Lúcio *et al.,* 2019) inducing proinflammatory processes (Campos-Múzquez *et al*., 2021) due to internalization in cells of the endometrial epithelium (Campos-Múzquez *et al*., 2019) causing abortions (Clune *et al*., 2022); *C. fetus* can also affect humans and has been reported to induce systemic infections (Adhikari *et al*., 2022) endocarditis (Lynch *et al*., 2022) and meningitis (Fernández *et al*., 2022). Bacteriological isolation and the use of biochemical tests play an important role for the identification of *C. fetus* and differentiation between *Cff* and *Cfv*, as they are considered the gold standard, however, the implementation of these culture techniques is difficult, as *Campylobacter* requires more demanding *in vitro* culture conditions than other bacterial genera, in addition, the culture is poor in reliability and reproducibility (Iraola *et al.,* 2016). Different methods can be used for *Cfv* identification, such as antigen-antibody reaction as Enzyme-Linked Immunoassays (ELISA), Direct Immunofluorescence (DI) (Dorsch *et al*., 2022) and molecular methods as Pulsed Field Gel Electrophoresis (PFGE), Amplified Fragment Length Polymorphism (AFLP), multilocus sequence typing and Polymerase Chain Reaction (PCR). Currently PCR is the test with the most promising alternative to efficiently detect *Cfv* from field samples (Chaban *et al.,* 2012). The objective of this study was to detect the presence of *C. fetus* by PCR in smegma from bulls used as breeders in the central zone of Tamaulipas state.

MATERIAL AND METHODS

Sampling

A convenience sampling was carried out in bovine stallions older than 2 years, of different breeds (Charolais, Beefmaster, red Brangus, Brahman) in Cattle Production Units (CPU) located in the central zone of Tamaulipas, Mexico. The study was carried out from August to December 2020. To obtain preputial samples, sterile disposable syringes and cannulas were used to deposit 60 mL of Ringer's Solution (PISA®) in the foreskin of each stallion; then the preputial orifice was closed and a vigorous massage was performed for 5 minutes

throughout the foreskin, subsequently the contents of the lavage (smegma) were obtained by draining and deposited in a sterile plastic bag with a hermetic seal. The semen samples were obtained by electroejaculation using minitube® equipment. The stimulation of the ejaculate did not exceed 20 volts per animal. For the collection of the ejaculate, a collection case was used, which holds a latex funnel, at the end of which a graduated tube was placed to receive the semen; this tube was identified before being used. The samples were kept refrigerated (4 °C) during transport and until processing (Silveira *et al.,* 2018).

DNA isolation

The collected samples were centrifuged at 604 xG for 5 min (Centurion Sc limited, UK) in sterile 50 ml plastic tubes, then the supernatant was discarded and the sediment was reconstituted with 0.2 ml of sterile 1X PBS. For genomic DNA isolation, all samples were treated with a commercial DNeasy blood and tissue kit (QIAGEN® Germany), following the manufacturer's instructions. Once the DNA collection process was completed, the genomic products were kept frozen (-20 ˚C) until further use. To evaluate DNA purification, a spectrophotometric study was performed at 260/280 nanometers (JENWAY Genova, UK), and ideal purity values were considered to be 1.8 to 2.0; DNA samples found to be in the range were subjected to DNA amplification by PCR.

DNA amplification and electrophoresis

A multiplex PCR was used; the primers used were nC1165g4F (AGGACACACAAATGGTAACTGG) and nC1165g4R (GATTGTATATAGCGACTTTGC) to detect a region of the *Cfv cstA* gene with amplification products of 233 base pairs (bp); primers MG3F (GGTAGCCGCAGCTAAGAT) and MG4R (TAGCTACACAATAACGACAAC) detect the *virB11 Cff* gene region amplifying to 764bp (Iraola *et al.,* 2012 y Hum *et al.,* 1997). As a positive control for the PCR reaction, nucleic acid from an ATCC reference strain of *Cff 27374* was used, subsequently when performing the PCR assays, a sample (sample 1) with amplification suggestive of *Cfv* was identified. The amplification result was sequenced by the Institute of Biotechnology of UNAM. The sequence was analyzed in the GenBank database using the BLAST online tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The PCR reaction was performed in 50 µL, (containing 5 µL of 10 X Buffer (Tris-HCl 100 mM pH 8.4, KCl 500 mM, gelatin 10 µg/ml, BSA 1.5 mg/ml, 1% Triton X 100 (Biotecmol, BIOTECMOL®, CDMX Mexico), dNTPS 0. 2 mM (InvitrogenTM, Life technologies; USA), 25 µM of each primer, MgCl2 3.0 mM (BIOTECMOL® CDMX, Mexico), 5 U of Taq DNA polymerase (Amplificasa® BIOTECMOL, CDMX, Mexico), plus 300 ng of DNA, finally adjusted with nuclease-free water to 50 µL. The PCR cycling conditions used were initial denaturation for 7 min at 95 °C followed by 35 cycles of denaturation for 30 sec at 94 °C, alignment 30 sec at 53 °C; and extension for 1 min at 72 °C, and a final extension of 2 min at 72 °C (Iraola *et al.,* 2012). The final

products were analyzed by 3% agarose gel electrophoresis, and then stained with ethidium bromide (promega) at a concentration of 0.5 µg/mL for 30 min. For visualization and identification of PCR products, a UV transilluminator was used and photodocumented on the E-gel imaging system. PCR products were purified with the commercial PureLink™ PCR Purification Kit (Invitrogen, Carlsbad, USA). Some PCR products were sequenced at the Institute of Biotechnology UNAM, Mexico City, Mexico. Sequencing results were analyzed in BLASTn. For sequence comparison, the NCBI/GenBank database was used (Altschul *et al.*, 2012).

RESULTS

Thirty-eight bulls were sampled; one sample was obtained from each; 31 from preputial lavage and 7 from semen. 18.4% were positive samples (7/38) (Figure 1), of these, two were from semen and five from preputial lavage. Of the 7 positive samples, 5 PCR products were subjected to sequencing. All sequences were compared considering the bacterium with accession number JF901335.1 in Genbank.

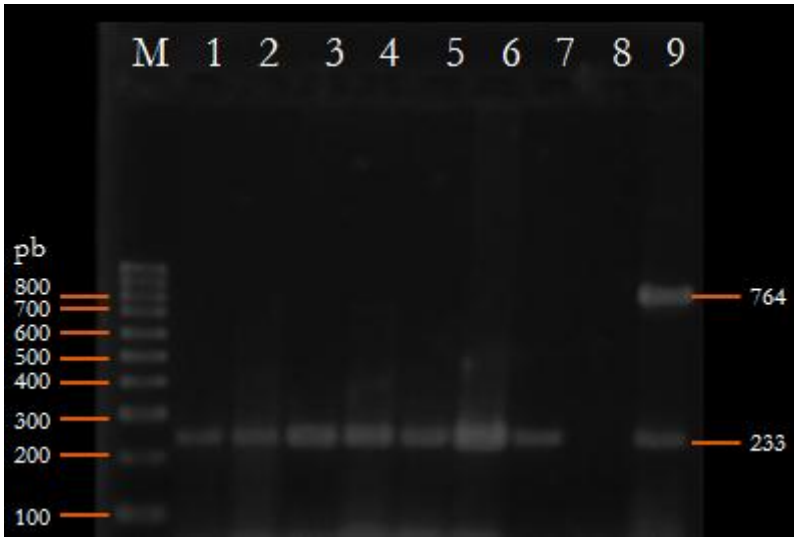

Figure 1. 2.5% agarose gel with the PCR products. Lane M: 100 mpb (BIOLINE, USA); lanes 1-9: Cfv positive; lane 8: negative control; lane 9: positive control *Cff 764* bp and *Cfv 233* bp

The nucleotide sequences of the amplicons obtained from field samples by PCR were identical to those found in the GenBank database. The analysis showed between 99.17 and 97.87% identity with other *Cfv* (Fig. 2). These results demonstrated that Cfv is present in herds in central Tamaulipas, and that *Cfv* plays an important role in the reproductive health of cattle.

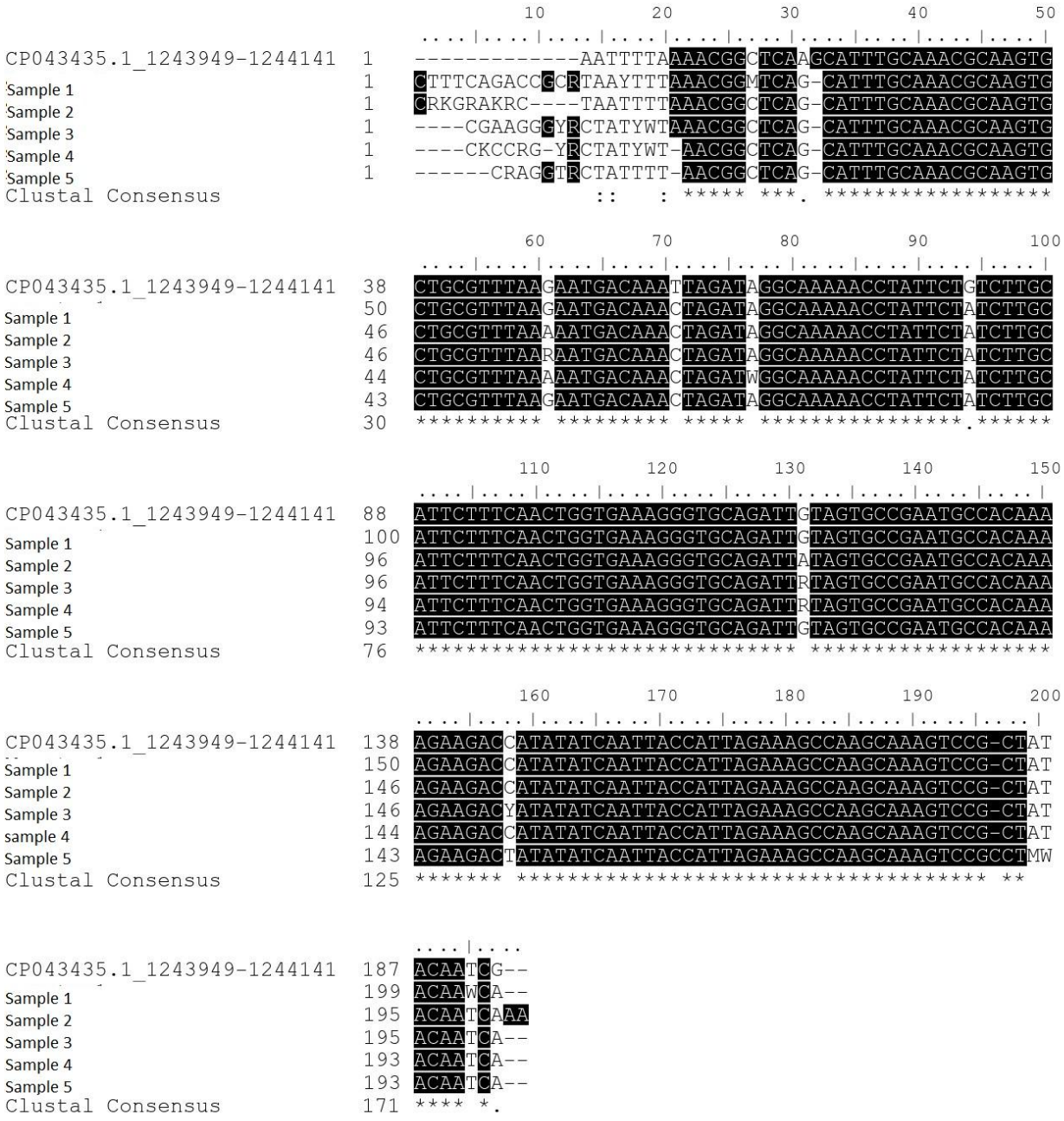

Figure 2. Alignment of *Cfv* sequences in bull samples from central Tamaulipas. EMBL-EBI Clustal Omega software was used

DISCUSSION

It is documented that BGC affects cattle herds; pathology caused by *Campylobacter fetus* (*C. fetus*) subspecies *venerealis* (*Cfv*). BGC is one of the main causes of infertility and abortion in cattle, sheep and goats; and its presence has been confirmed in several countries of the American continent such as Argentina, Brazil, Costa Rica, Jamaica and Mexico. However, in Mexico, this pathogen has been little studied in livestock areas. The detection of this pathogenic subspecies in bulls used as sires is of utmost importance for the control of this disease in herds, since they are asymptomatic carriers; and, in addition to being used for direct mating, semen is frequently used for artificial insemination, both fresh and in straws preserved in liquid nitrogen. In addition to infertility problems, *Cff* is considered the main cause of sperm alterations due to the ability of these bacteria to adhere to the spermatozoa causing damage to the acrosomal membrane as well as to the sperm chromatin, which decreases semen quality and consequently the pregnancy percentage, besides influencing infertility in females due to the transmission of bacteria to the genital tract during natural mating (Cagnoli *et al.,* 2020).

The diagnosis of this disease can be performed by various methods such as bacteriological isolation, serological methods such as ELISA and DI, and molecular assays such as PCR (Mshelia *et al.,* 2010; Clune *et al*., 2022). Bacteriological isolation is the least sensitive method, as demonstrated by Groff *et al.,* en 2010, where they compared this method with PCR, resulting in this molecular method being 8.5 times more sensitive than traditional bacteriological isolation. In this study, PCR was used following the recommendations of other researchers; for the identification of *Cfv* with the *cstA* gene and for *Cff* with the *virB11* gene (Hum *et al.,* 1997; Iraola *et al*., 2012), given that the primers generated for the identification of these genes can detect specific DNA sequences of *Cfv* and *Cff*; with the PCR developed allowed positive cases of CGB to be detected in the study area. Another method that has been used is DI; however, Ferreira en 2002 shows that the technique does not distinguish *C. fetus* subspecies.

In Mexico, beef cattle have been exported to the United States of America since 1994, and the state of Tamaulipas has been characterized for having one of the largest inventories in the country. In the 2020-2021 cycle, the export of live cattle was 30,896 head to the United States according to data from the Secretariat of Agriculture and Rural Development (SADER). Considering the need to increase the number of animals to supply the demand, it is necessary to have an animal health diagnosis of diseases that affect reproductive parameters. The success of the diagnosis depends largely on sampling; based on previous reports reported by Silveira *et al.,* 2018 and Delpiazzo *et al.,* 2021.

The prevalence of BGC is variable in different areas of the world, and there are several factors that favor its transmission (Hoque *et al.,* 2021). In Mexico, little has been reported on the presence of *Campylobacter* in cattle; the only study found in our country was conducted by Barajas en 2013 in tropical areas of Mexico; by means of ELISA test, it

revealed a seroprevalence of 21.3% in cattle. The detection method was different and the sampling was lower; the frequency detected in this study was 18.4%, indicating that BGC is present in Tamaulipas cattle herds. It is worth mentioning that Barajas' work was carried out with cattle from the humid tropics of the extension program for cattle ranchers in northern Veracruz, a state adjacent to Tamaulipas and with climatic conditions similar to the study area.

The objective of this study was to detect the presence of *C. fetus* in bulls from the central zone of Tamaulipas state. From the 7 amplifications suggestive of *Cfv* by PCR, 5 were evaluated by sequencing. The results obtained in the bioinformatic analysis confirm that the amplification corresponds to a region of the *virB11* gene which is a sequence of pathogenicity islands present only in *Cfv* with 233 bp and absent in *Cff,* which allows differentiating these two subspecies by molecular method. The results obtained in the present work are consistent with what was previously published by Iraola *et al.,* 2016, so the epidemiological situation could be at risk due to the spread of this pathogen by the use of carrier bulls for reproductive purposes.

CONCLUSION

In this study, the presence of *Campylobacter fetus* subsp. *venerealis* was detected by PCR and corroborated by sequencing with a frequency of 18.4% of the bulls analyzed from the central zone of Tamaulipas state, which represents the first report in Mexico of this bacterium by molecular methods.

ACKNOWLEDGMENTS

To ZVD. Francisco Trejo Meza from The Tamaulipas Regional Livestock Association's Genetic Improvement Center for allowing the sampling of milking bulls.

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Errata Erratum
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