

***Campylobacter* sp. IN DAIRY COWS FROM SOUTHERN BRAZIL**

(*Campylobacter* sp. em vacas leiteiras do Sul do Brasil)

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ABSTRACT: The present study aimed at assessing the occurrence of *Campylobacter jejuni*, *Campylobacter coli*, and *Salmonella enterica* in dairy cows and milking parlors from dairy farms in south Brazil. Samples were collected from 12 dairy farms located in the southern of the Rio Grande do Sul State. During the visits, one sample of the water used in the milking parlor were collected, also one sample of the milking machines, one of the inner surfaces of the milk pails or pipe walls, one from the handler's hands, and one from mixed milk were collected for microbiological analysis. Feces from five cows in production were also sampled and tested for the presence of *Campylobacter* and *Salmonella*, totaling 10 samples from each dairy farm. *Salmonella* was not isolated from any of the samples analyzed. *Campylobacter* was isolated from fecal samples of three cows (3/60; 0.05%) from the same farm. One of these isolates was identified as *C. jejuni* which has the *cdt* gene coding for the cytolethal distending toxin. We conclude that *Campylobacter* sp. may be found in dairy cattle during the milking process. Infected animals shed this microorganism into the environment through their stools. Therefore, there is a risk of transmission of this pathogen to humans by direct or indirect contact with the feces of these cows.

Key words: milk, milking environment, public health, *Salmonella*.

RESUMO: O presente estudo teve como objetivo avaliar a ocorrência de *Campylobacter jejuni*, *Campylobacter coli* e *Salmonella enterica* em vacas leiteiras e salas de ordenha de fazendas leiteiras no sul do Brasil. Foram coletadas amostras de 12 fazendas leiteiras localizadas no sul do Rio Grande do Sul. Durante as visitas às propriedades, foram coletadas amostras da água utilizada na sala de ordenha, dos insufladores das ordenhadeiras mecânicas, da superfície interna dos baldes de leite ou da parede da tubulação, das mãos dos manipuladores e do leite de conjunto foram coletadas para as análises microbiológicas. Também foram coletadas amostras de fezes de cinco vacas em

produção, as quais também foram avaliadas quanto à presença de *Campylobacter* e *Salmonella*, totalizando 10 amostras em cada fazenda leiteira. Não houve o isolamento de *Salmonella* em nenhuma das amostras que foram analisadas. *Campylobacter* foi isolado de três amostras (3/60; 0,05%) de fezes de vacas de uma mesma propriedade, sendo um dos isolados identificado como *C. jejuni*, esse isolado apresentou o gene *cdt* que codifica para a toxina citoletal distensiva. Conclui-se com o estudo que *Campylobacter* sp. pode ser encontrado em bovinos leiteiros em processo de ordenha, podendo oferecer risco de transmissão desse micro-organismo pelo contato direto ou indireto com as fezes dos animais contaminados.

Palavras-chave - ambiente de ordenha, leite, *Salmonella*, saúde pública.

INTRODUCTION

The microbiological quality of milk is related to hygienic sanitary practices during its production. It influences the nutritional properties of milk and its derivatives and is fundamental for the safety of the consumers (Beloti, 2015).

Milking is an important step to produce milk products of high quality. Factors related to the hygiene of milking equipment, environment, and handlers may lead to milk contamination and have public health implications raising the risk of human infection and the occurrence of foodborne diseases (Miguel et al., 2012). The identification of the main sources of contamination for different pathogens that can be transmitted to humans by the ingestion of contaminated food stands out as a useful tool to improve milk quality and prevent foodborne diseases (Kuaye and Passos, 2017).

Salmonella and *Campylobacter* are important microorganisms in public health and are the bacteria most frequently reported worldwide as causes of foodborne illness in 2016 according to data published by the Centers for Disease Control and Prevention (CDC) (Marder et al., 2018). In Brazil, *Salmonella* sp. was the second most identified microorganism in foodborne outbreaks from 2009 to 2018, after *Escherichia coli* (Brazil, 2019). On the other hand, foodborne illness caused by *Campylobacter* is underestimated in Brazil because this microorganism is not routinely tested in foodborne disease investigations (Bessa et al., 2020).

Campylobacter is the bacterium that causes the largest number of cases of diarrhea in developed and developing countries (Platts-mills et al., 2015). The cytolethal distending toxin (CDT) which is encoded by the *cdt* gene is an important pathogenicity factor of *C. jejuni* (Young et al., 2007). This toxin causes an increase in cellular cyclic adenosine monophosphate (cAMP) and imbalances in the mechanisms of absorption and

secretion of electrolytes into the intestinal lumen, leading to profuse and watery diarrhea (Guiffrida, 2016). Milk and derivatives are among the foods involved in outbreaks of food toxoinfection caused by *Campylobacter* (Hunt et al., 2009). In addition to the enteric infection, *C. jejuni* infection has been associated with Guillain-Barré syndrome which is a serious illness characterized by acute neuromuscular paralysis (Brasil, 2015).

Salmonella is widely distributed in nature especially in the intestinal tract of animals and humans (Koneman et al., 2018) and is easily spread in the environment as this bacterial pathogen is spread through feces. It is important to understand the epidemiology of the microorganism to prevent toxoinfections in humans through food consumption (Tahergorabi et al., 2012) especially milk and its derivatives (CDC, 2015).

There are few published studies on *Salmonella* and *Campylobacter* in the milking environment in south Brazil. Therefore, further research regarding the presence of these pathogens at the milking site is needed.

The present study aimed to assess the occurrence of *C. jejuni*, *C. coli*, and *S. enterica* in dairy cows and milking parlors of dairy establishments in southern Brazil and to screening for the *cdt* gene in *Campylobacter* isolates.

MATERIAL AND METHODS

Sample collection

The present survey consisted of one collection that was carried out in 12 rural dairy farms located in the southern part of the State of Rio Grande do Sul. In each of these premises, between 10 and 70 cows were milked mechanically twice a day with a piped system, with a bucket at the foot, or manually. These facilities were equipped with a direct expansion tank to cool the milk. During the visits, one sample of the water used in the milking parlor were collected, one sample of the milking machines, one of the inner surfaces of the milk pails or pipe walls, one from the handler's hands, and one from mixed milk were collected for microbiological analysis. Fecal samples were also collected from 5 cows in production, totaling 10 samples from each dairy farm. After visiting the 12 establishments, 120 samples were obtained in total.

Sterilized swabs were used to collect samples of the handler's hands, equipment, and utensils. These samples were collected prior to the start of the milking process. The technique recommended by the American Public Health Association (APHA, 2001) was used to collect samples from the hands of the milkers. The swab was rubbed in rotational movements from the lower right palm to the tip of the toes. This procedure was repeated three times towards each finger. The swab was passed on the edges of

handler's hands in forward and backward strokes on one side of the hand in which the wrist line begins, moving between the fingers and ending on the other side of the hand to the wrist. For the bucket surface sampling, the swab was rubbed on an area of 100 cm² at the bottom of the bucket which was delimited by a sterile stainless-steel jig (APHA, 2001). For the sampling of the pipe walls, swabs were rubbed over the inner surface of these pipes and along the entire extent of these tubular structures up to a 10 cm height. Samples from the insufflators were collected by rubbing the swabs in circular movements in the terminal segment of each of these devices (Tavares et al., 2017).

Samples of the water used for the different production and hygiene activities in the facilities were aseptically collected directly from the taps. After allowing these samples to flow for 3 min, an approximate amount of 400 mL of each specimen was poured into sterile glass vials. In the properties with water treatment, 0.1 mL of sodium thiosulfate in 10% solution per 100 mL of each sample was used to neutralize the action of chlorine (APHA, 2001).

For the sampling of raw milk, the collection was performed directly from the refrigeration tank after homogenization. With the aid of a sterilized spoon, 200 mL samples were collected and stored in sterile glass vials (Tavares et al., 2017).

Fecal samples from cows were collected from the rectum using swabs. After sampling, these swabs were placed in Cary Blair transport medium (Himedia, India). Specimens were then packed in isothermal boxes containing recyclable ice and immediately sent to the laboratory for microbiological analysis.

The producers whose farms were included in this study agreed to sign a consent form allowing their premises to be included in the survey.

Isolation of Campylobacter

For the isolation of *Campylobacter* from milk and water, 25 mL aliquots of the sample were added to 225 mL of *Brucella* broth (Acumedia, USA) containing 0.4% (w/v) activated charcoal, 5% (m/v) supplementation of FBP oxygen reducing solution (George et al., 1978) and 1% (w/v) *Campylobacter* I supplement (Blaser-Wang) (Himedia, India) which contains antibiotics to control the growth of the accompanying microbiota. After sample incubation at 42°C for 48h in a microaerophilic atmosphere (85% N₂, 10% CO₂, and 5% O₂), an aliquot of this broth was seeded on the surface of Columbia Blood Agar Base (Acumedia, USA) with the same additions of *Brucella* broth. The swabs from the surfaces, hands, and stool samples were incubated in tubes with 10 mL of *Brucella* broth with the same additions and for the same incubation period of the milk and water

samples and were later seeded on the surface of the Columbia Blood Agar Base medium. The plates were incubated at 42°C for 48 h under a microaerophilic atmosphere. The morphotinctorial characteristics of typical scattered colonies producing a water glow were studied using the Gram staining. Colonies of bacteria with typical "S" shape or gull wing morphology were cryopreserved in stock medium (1g neopepton, 25 mL glycerol, 0.5g NaCl, and 75 mL distilled water) and recovered if needed.

Isolation of Salmonella

For the *Salmonella* detection, 25 mL of the milk and water samples were added to 225 mL of Buffered Peptone Water (APT, Acumedia, USA). The swabs from the surfaces, hands, and stool samples were placed in test tubes with 10 ml APT. Then, 0.1 ml of the mixture was transfer to 10 ml Rappaport-Vassiliadis (RV, Himedia, India) medium and another 1 ml mixture to 10 ml tetrathionate (TT, Himedia, India) broth. RV medium and TT broth were incubated for 24 ± 2 h at 41 ± 0.2 °C. Enriched aliquots (10 µl) were then streaked onto Xylose Lysine Deoxycholate (XLD, Merck, Germany) and Brilliant-green phenol-red lactose sucrose agar (BPLS, Merck, Germany) and incubated at 37°C for 24h. Presumptive *Salmonella* species were confirmed using biochemical (triple sugar iron, lysine iron agar, urease, and iodole production) and serological (*Salmonella* O Poly A-I & Vi Antiserum, Difco, USA) methods.

DNA extraction

The DNA of the isolates was extracted according to Sambrook and Russel (2001). Briefly, the pellet obtained by centrifugation of 1 ml of the bacterial culture was resuspended in 100 µL of STES buffer [0.2 M Tris-HCl, 0.5M NaCl, 0.1% SDS (w/v), EDTA 0, 01M, pH 7.6]; 50 µL of glass beads and 100 µL of phenol/ chloroform were added to these pellets. After homogenization for 1 min, the mixture was centrifuged at 13,000 g for 5 min. The supernatant was collected and precipitated in 2 volumes of absolute ethanol and 0.1 volume of 5 M NaCl at -70°C for 30 min. An additional centrifugation was performed at 13,000 g for 20 min. The supernatant was discarded, and the pellet washed with 70% ethanol. After elution in 40 µL of elution buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4), 1 µl of RNase (10 µg / µL) was added to the pellet.

Polymerase chain reaction

DNA from the *Campylobacter* isolates was analyzed by the polymerase chain reaction (PCR) technique as described by Harmon et al. (1997) for the differentiation between *C. jejuni* and *C. coli* species. Two primer pairs were used in this PCR analysis (Table 1). Pair I (pg 3 and pg 50) amplify a highly conserved region related to flagellin genes in both *C. jejuni* and *C. coli*.

Pair II (C-1 and C-4) amplifies a specific region that is present only in *C. jejuni*. Each reaction had a final volume of 25 μ L; 12 μ L of Master Mix (Promega, USA), 2 μ L (20 pmol) of each primer, 1 μ L DNA (at 5 nmol μ L⁻¹ concentration), and 4 μ L DNA-free water were used to complete the reaction. The amplification was performed in TC-3000 (Techne) thermocycler with the following program: initial denaturation of 94°C for 4 min followed by 25 cycles of denaturation at 94°C for 1 min, annealing of the primers at 45°C for 1 min, extension at 72°C for 1 min, and final extension at 72°C for 7 min. The analysis of GelRed-stained amplifications (Uniscience, Brazil) was done on 1.5% agarose gel electrophoresis.

Table 1 - Primers used in the differentiation between *C. jejuni* and *C. coli*.

Primer	Sequence (5' to 3')	Species	Size of PCR amplification (pb)
Pg 3	GAACTGAACCGATTTG	<i>C. coli</i>	460
Pg 50	ATGGGATTTTCGTATTAAC	<i>C. jejuni</i>	460
C-1	CAAATAAAGTTAGAGGTAG.AATGT	<i>C. jejuni</i>	160
C-4	GGATAAGCACTAGCTAGCTGAT		

The multiplex PCR technique was performed for the identification of the *cdt* genes of *C. jejuni* according to the protocol published by Martinez et al. (2006) using specific primers for the *cdtA*, *cdtB*, and *cdtC* genes (Table 2).

Table 2 - Primers used to search for the *cdtA*, *cdtB*, and *cdtC* genes of *C. jejuni*.

Primer	Sequence (5' to 3')	Size of PCR amplification (pb)
<i>cdtA</i> -F	CTATTACTCCTATTACCCACC	422
<i>cdtA</i> -R	AATTTGAACCGCTGTATTGCTC	
<i>cdtB</i> -F	AGGAACTTTACCAAGAACAGCC	531
<i>cdtB</i> -R	GGTGGAGTATAGGTTTGTGTC	
<i>cdtC</i> -F	ACTCCTACTGGAGATTTGAAAG	339
<i>cdtC</i> -R	CACAGCTGAAGTTGTTGTTGGC	

Ethics Committee approval

This research proposal was approved by the Ethics Committee on Animal Experimentation of the Federal University of Pelotas (UFPel, Pelotas, RS, Brazil) under the accession number (ID number) EAEC 5134-2015.

RESULTS AND DISCUSSION

Salmonella was not isolated from any of the samples. Generoso and Langoni (2011) investigated eight dairy farms located in the state of São Paulo, southeast Brazil, and *Salmonella* was not isolated from any of the cattle, milking environment, or raw milk samples.

Campylobacter was isolated from fecal samples of three cows (3/60; 0.05%) from the same dairy farm. However, this microorganism was not isolated from other sampling sites. The isolate from cow 1 was identified as *C. jejuni* whereas the isolates from the other two cows were classified as *Campylobacter* sp. Molecular identification of the species was not possible since this is a fastidious microorganism and is therefore difficult to grow.

The presence of infected cows in a dairy herd facilitates the spread of *Campylobacter* in the premise. In 2014, an outbreak of campylobacteriosis occurred after preschool students visited a dairy farm in Sweden. During the visit, a meal including unpasteurized milk was served. Of the 30 students tested, 11 were infected with a foodborne pathogen. *C. jejuni* was isolated from eight of these individuals. An epidemiological investigation on the facility was carried out. *Campylobacter* was isolated from cows on this farm where this outbreak of food poisoning occurred. Genotyping with pulsed field electrophoresis and genetic sequencing showed a similarity between the *C. jejuni* isolates from humans and from cattle sampled. Thus, livestock at the farm was considered the source of infection. The most likely vehicle transmission in these cases was contaminated unpasteurized milk (Lahti *et al.*, 2017).

Evans *et al.* (1996) carried out a survey on cases of gastrointestinal infection caused by *Campylobacter* in a group of children shortly after they visited a farm and ate snacks at that premise. These children consumed raw milk possibly contaminated with feces from dairy cows shedding bacterial pathogens in their stools. In two additional visits from other groups of children from the same school, there were no reports of gastroenteritis even though they had consumed raw milk.

Our results demonstrate that although in this study *Campylobacter* was not detected in milk samples, there is still a risk of transmission of a bacterial agent shed in the feces of cows to both the milk and the milker if the hygienic and sanitary measures are inadequate.

The *cdt* genes were detected in the isolate from cow 1 had and therefore this isolate has the potential to produce toxins. Our findings agree with those from other studies published elsewhere (Martinez *et al.*, 2006; Silva *et al.*, 2014) which demonstrated

that most *C. jejuni* strains have *cdt* genes. Therefore, most of the isolates are from *C. jejuni* toxin-producing strains.

CONCLUSION

Campylobacter sp. may be found in dairy cattle during the milking process. The presence of this bacterial pathogen in the milking environment poses a risk of *Campylobacter* transmission by direct or indirect contact with the feces of infected animals that shed this microorganism into their stools.

The finding of *Campylobacter* in samples of raw milk indicates the need to implement adequate and effective measures for the control and elimination of this pathogen from the production chain as well as the heat treatment of milk destined for human consumption.

REFERENCES

- ANDREWS, W.H; WANG, H.; JACOBSON, A. et al. *Salmonella*. U.S. Food and Drug Administration. **Bacteriological analytical manual online (BAM)**, 2016. Available at: <<https://www.fda.gov/food/foodscienceresearch/laboratorymethods/ucm070149.htm>>. Accessed in: april 2018.
- AMERICAN PUBLIC HEALTH ASSOCIATION (APHA). **Compendium of methods for the microbiological examination of foods**. Washington: APHA Press, 2001. 676 p.
- BELOTI, V. **Leite: obtenção, inspeção e qualidade**. Londrina: Editora Planta, 2015. 420 p.
- BRASIL. Ministério da Saúde. Secretaria de Atenção à Saúde. **Portaria nº 1171, de 19 de novembro de 2015**. Available at: <<http://portalarquivos2.saude.gov.br/images/pdf/2015/novembro/20/MINUTA-de-Portaria-SAS-PCDT-Guilain-Barr---ATUALIZADO-11-11-2015.pdf>>. Accessed in april 2018.
- BRASIL. Ministério da Saúde. Secretaria de Vigilância em Saúde. Departamento de Vigilância de Doenças Transmissíveis. **Surtos de doenças transmitidas por alimentos no Brasil – Informe 2018**. Available at: <<https://portalarquivos2.saude.gov.br/images/pdf/2019/fevereiro/15/Apresenta----o-Surtos-DTA---Fevereiro-2019.pdf>>. Accessed in: december 2020.
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CENTERS FOR DISEASE CONTROL AND PREVENTION (CDC). *Salmonella*, 2015. Available at: <<http://www.cdc.gov/salmonella/>>. Accessed in: april 2018.

EVANS, M.R.; ROBERTS, R.J.; RIBEIRO, C.D. et al. A milk-borne *Campylobacter* outbreak following an educational farm visit. **Epidemiology & Infection**, v.117, n.3, p.457-462, 1996.

GEORGE, H.A.; HOFFMANN, P.S.; KRIEG, N.R. et al. Improved media for growth and aerotolerance of *Campylobacter fetus*. **Journal of Clinical Microbiology**, v.8, n.1, p.36-41, 1978.

GENEROSO, D.; LANGONI, H. Avaliação da presença de *Salmonella* sp. na criação de bovinos de leite. **Veterinária e Zootecnia**, v.18, n.4, p.661-667, 2011.

GUIFFRIDA, R. Infecções pelo gênero *Campylobacter*. In: MEGID, J.; Ribeiro, M.G.; Paes, A.C. **Doenças infecciosas em animais de produção e de companhia**. Rio de Janeiro: Roca, 2016, Cap.33, p.347-355.

BESSA, M.C., HAAS, S., RAMOS, R.C., et al. Survey of *Campylobacter* in foods implicated in foodborne diseases in Southern Brazil. **Revista do Instituto de Medicina Tropical de São Paulo**, 62, 2020.

HARMON, K.M.; RANSOM, G.M.; WESLEY, I.V. Differentiation of *Campylobacter jejuni* and *Campylobacter coli* by polymerase chain reaction. **Molecular and Cellular Probes**, v.11, n.3, p.195-200, 1997.

HUNT, D.C.; BAÑEZ, M.C.; NEISES, D. et al. *Campylobacter jejuni* infection associated with unpasteurized milk and cheese. **Morbidity and Mortality Weekly Report**, v.57, n.51, p.1377-1379, 2009.

KONEMAN, E.W.; ALLEM, S.D.; JANDAW, M. et al. **Diagnóstico microbiológico - Texto e atlas colorido**. Rio de Janeiro: Medsi, 2018. 1760 p.

KUAYE, A.Y. **A higienização e a segurança de alimentos**. Rio de Janeiro: Atheneu, 2017. 323 p.

LAHTI, E.; REHN, M.; OCKBORN, G. et al. Outbreak of campylobacteriosis following a dairy farm visit: confirmation by genotyping. **Foodborne pathogens and disease**, v.14, n.6, p.326-332, 2017.

MARDER, E.P.; GRIFFIN, P.M.; CIESLAK, P.R. et al. Preliminary Incidence and Trends of Infections with Pathogens Transmitted Commonly Through Food—Foodborne Diseases Active Surveillance Network, 10 US Sites, 2006–2017. **Morbidity and Mortality Weekly Report**, v.67, n.11, p.324-328, 2018.

MARTINEZ, I.; MATEO, E.; CHURRUCA, E. et al. Detection of *cdtA*, *cdtB*, and *cdtC* genes in *Campylobacter jejuni* by multiplex PCR. **International Journal of Medical Microbiology**, v.296, n.1, p.45-48, 2006.

MIGUEL, P.R.R.; POZZA, M.S.S.; CARON, L.F. et al. Incidência de contaminação no processo de obtenção do leite e suscetibilidade a agentes antimicrobianos. **Semina: Ciências Agrárias**, v.33, n.1, p.403-416, 2012.

PLATTS-MILLS, J.A.; BABJI, S.; BODHIDATTA, L. et al. Pathogen-specific burdens of community diarrhoea in developing countries: a multisite birth cohort study (MAL-ED). **The Lancet Global health**, v.3, n.9, p.564-575, 2015.

SAMBROOK, J.; RUSSEL, D.W. **Molecular Cloning: A Laboratory Manual**. New York: Cold Spring Harbor Laboratory Press, 2001. 2028 p.

SILVA, D.T.; TEJADA, T.S.; CUNHA, C.C. et al. Occurrence of *Campylobacter* in poultry, meat chicken and human feces, and *cdt* genes research. **Arquivo Brasileiro de Medicina Veterinária e Zootecnia**, v.66, n.1, p.297-304, 2014.

TAHERGORABI, R.; MATAK, K.E.; JACZYNSKI, J. Application of electron beam to inactivate *Salmonella* in food: Recent developments. **Food Research International**, v.45, n.2, p.685–694, 2012.

TAVARES, A.B.; SOUZA, A.I.A.; DULAC, C.F. et al. Fontes de contaminação de *Yersinia enterocolitica* durante a produção de leite. **Arquivo Brasileiro de Medicina Veterinária e Zootecnia**, v.69, n.2, p.483-490, 2017.

YOUNG, K.T.; DAVIS, L.M.; DIRITA, V.J. *Campylobacter jejuni*: molecular biology and pathogenesis. **Nature Reviews**, v.5, n.9, p.665-679, 2007.