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Anaplasma marginale transplacental transmission in a calf: clinical, anatomopathological, and molecular reports

Transmissão transplacentária de *Anaplasma marginale* em bezerra: relatos clínicos, anatomopatológicos e moleculares

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RESUMO

A anaplasmose bovina é causada por *Anaplasma marginale*, uma riquétsia intracelular obrigatória, transmitida por carrapatos *Rhipicephalus microplus*, moscas hematófagas e/ou iatrogenicamente. A transmissão transplacentária *Anaplasma marginale* também pode ocorrer, mas é subnotificada. Neste contexto, o presente trabalho teve como objetivo relatar o caso de uma bezerra que nasceu com anemia, icterícia e problemas neurológicos. Um esfregaço de sangue da bezerra mostrou inclusão intraeritrocítica de *A. marginale*. Assim, o animal foi tratado com oxitetraciclina, mas foi a óbito, três dias após o início do tratamento. A necrópsia revelou icterícia conjuntival e subcutânea, congestão e distensão esplênica, além de fígado intensamente amarelo. A análise histopatológica mostrou degeneração hepatocelular multifocal moderada, colestase difusa e acentuada e encefalite necrosante histiocítica. A infecção por *A. marginale* foi confirmada por PCR e PCR em tempo real (qPCR). A análise por qPCR mostrou que 3,3% dos eritrócitos da amostra de sangue da bezerra estavam parasitados. Assim, confirmamos e relatamos nesse estudo um caso de transmissão transplacentária de *A. marginale* em uma bezerra recém-nascida.

Palavras-chave: Anaplasmose; Bovino; Anaplasmose.

ABSTRACT

Bovine anaplasmosis is caused by obligate intracellular rickettsia *Anaplasma marginale* transmitted by ticks *Rhipicephalus microplus*, hematophagous flies, and/or iatrogenically. Transplacental transmission is possible but sub-reported. In this context, the present work aimed to report a case of a calf that was born with anemia, jaundice, and neurological problems. A calf blood smear showed intraerythrocytic inclusion of *A. marginale*. Accordingly, the animal was treated with oxytetracycline but died three days after the start of treatment. The necropsy revealed conjunctival and subcutaneous jaundice, splenic congestion and distension, and an intensely yellow liver were noted. Histopathology showed moderate, multifocal hepatocellular degeneration, diffuse and marked cholestasis, and histiocytic necrotizing encephalitis. *A. marginale* infection was confirmed by PCR, and real-time PCR (qPCR). qPCR analysis showed that 3.3% of the erythrocytes in the calf's blood sample were parasitized. Thus, here we report a case of transplacental transmission *A. marginale* in a newborn calf.

Keywords: Anaplasmosis; Bovine; Congenital Anaplasmosis.

INTRODUÇÃO

Bovine anaplasmosis, a tick-borne disease caused by the mandatory intracellular pathogen *Anaplasma marginale*, is responsible for high morbidity and mortality in cattle (Guarnizo et al., 2020; Hove et al., 2020). It is transmitted mainly by ticks, mosquitoes, flies, and transplacentally (Atif et al., 2021). Transplacental transmission occurs when cows have *A. marginale infection* in chronic form, and positive calves may become persistently infected (Aktas and Özübek, 2017). The death of infected calves by transplacental route was previously reported (Costa et al., 2016; Henker et al., 2020; Pypers et al., 2011; Santarosa et al., 2013), highlighting the epidemiological importance of transmission (Aktas and Özübek, 2017).

Reports describing clinical signs due to congenital infections are scarce and show mainly anemia associated with apathy, dehydration, jaundice and respiratory problems (Girardi et al., 2012) However, such signs may contribute to the epidemiology of this disease in some areas and generate significant losses when not diagnosed in the herd, since the animals are asymptomatic and will only be identified by molecular tests, such as polymerase chain reaction (PCR) (Añez-Rojas et al., 2010; Kocan et al., 2010; Kovalchuk et al., 2020).

The most used tests to identify *A. marginale* in infected animals are microscopic tests of blood smears and serological (Kocan et al., 2010). However, conventional PCR and real-time PCR represent the most effective, fast, and sensitive alternatives for laboratory diagnosis (Kovalchuk et al., 2020; Reinbold et al., 2010). In the present study, we aimed to confirm by PCR and real-time PCR a case of anaplasmosis in a calf that was born with anemia, jaundice, and neurological problems.

MATERIALS AND METHODS

Case presentation

A female calf, three-days-old, weighing 30kg, presenting neurological disorders since birth was attended at the Veterinary Hospital of Uberaba University (HVU). It was initially treated for leptospirosis (Penicillin and Streptomycin-Diclopen® - 1mL/12.5Kg - 2.4 mL, IM, SID), Vitamin B1 (Monovim® B1- 2 mL, IM, SID), hepatic protector

(Mercepton® -10 mL, IM, SID) and was subsequently received in HVU because it did not improve with the recommended treatment.

The anamnesis revealed regular nutritional status, weak biotype, lateral decubitus, icteric mucosa, degree of dehydration 10%, capillary refill time (CRT): 5, respiratory rate: 52 breaths per minute (bpm), heart rate: 100 beats per minute (bpm), and temperature: 37.5ºC. Besides, hematological, biochemical and hemogasometric tests were performed by use of ortho vitro biochemical testing equipment 5600® (Ortho). Bloodstains, staining, and fixation of the cells were performed with the panoptic kit solutions (Laborclin®, Brazil).

The veterinarian prescribed Oxytetracycline (20mg/kg) (Tetroxy 200 L.A® - 3 mL, IM, SID), dexamethasone (0,07mg/kg) (Dexaflan® 2 mL, IM, SID), Vitamin B12 (Monovim B12® - 3 mL, IM, SID) and Fluid Therapy (Ringer lactate and glucose 5% – 6L/day, IV), but the animal died after three days of hospitalization. Subsequently, a necropsy and histopathological analysis were performed. Liver, spleen, heart, lung, and brain samples were collected and fixed in 10% neutral buffered formalin, processed routinely, and embedded in paraffin wax. Sections were stained with haematoxylin and eosin (HE).

Extraction of genomic DNA, PCR, and real-time PCR (qPCR)

A volume of 400 µL of calf blood sample was aliquoted into a 2 mL microcentrifuge tube, to which 1.5 mL of RBC Lysis Buffer (1.7 M NH_4Cl; 0.1 M $KHCO_3$; and 1 mM EDTA) was added. This mixture was homogenized by inversion, allowed to stand for 10 min at room temperature, and was then centrifuged for 10 min at 600 g. The supernatant was discarded, and the pellet was eluted in Cell Lysis Buffer (10 mM Tris-HCl, pH 8.0; 10 mM EDTA, pH 8.0; 50 mM NaCl; 10% SDS, pH 7.5; and proteinase K 10 mg/mL). The sample was incubated in a water bath at 56 °C for about 2 hours. Then equilibrated phenol with Tris-HCl pH 8.0 was added, homogenized by inversion and then centrifugated for 10 min at 10000 xg. Genomic DNA was extracted using a mixture of phenol, chloroform, and isoamyl alcohol. RNA was removed by adding 10mg/mL RNase A. Genomic DNA was precipitated with 100% ethanol. The pellet was washed with 70% ethanol, resuspended in nuclease-free water and quantifiedusing Qubit

dsDNA BR Assay (Invitrogen™). The DNA sample (9.4 ng/µL) was stored at -20 °C to perform a PCR diagnostic, later on.

The PCR for research of *A. marginale* in blood cells was performed for the 16 S ribosomal gene. The DNA was amplified using the oligonucleotide specific for the *Anaplasma* gene, ECC (5'-GAACGAACGCTGGCGGCAAGC-3') and ECB (5'-CGTATTACCGCGGCTGCTGGCA -3'), and non-specific primers ECAN (5'-CAATTATTTATAGCCTCTGGCTATAGGA-3') and HE3 (5'-TATAGGTACCGTCATTATCTTCCCTAT -3') (Faria et al., 2010) . For PCR, 25 µL of the reaction mixture was prepared, which consisted of 10× reaction buffer complete (Cellco), 0.2 µM of each dNTP, 0.2 µM of each primer, 0.625 U Taq DNA Polymerase (Cellco), 1 µL of target DNA. The cycling conditions for PCR were initial denaturation at 95 °C for 3 min followed by 35 amplification cycles (95 °C for 30 s, 60 °C for 30 s, 72 °C 30 s) and a final extension step at 72 °C for 2 min. The negative control contained all reaction components, except the template DNA.

The real-time PCR was performed on StepOne™ Real-Time PCR System Thermal Cycling Block (Applied Biosystems) with GoTaq® qPCR Master Mix (Promega, Madison, WI, United States). The 10 µL of reaction mixture contained 5 µL of GoTaq® qPCR Master Mix, 0.1 µL of CXR Reference Dye, 0.2 µM of each primer (ECC and ECB) and 1 µL of DNA from a calf. The thermocycling conditions consisted of a denaturation step at 95°C for 10 min, followed by 40 cycles of a denaturation step at 95°C for 30 s, annealing at 60 °C for 1 min. After amplification, a dissociation curve was generated to verify the specificity of amplification. The melting program started with denaturation at 95 ◦C for 30 s, annealing at 60 ◦C for 1 min, followed by 1.0 ◦C temperature increase every 30 s until 95 ◦C. The real-time PCR was carried out in triplicate and a no-template control was included in the assay. The increase in fluorescent signal was registered at the extension step of the reaction and the data were analyzed with the StepOne™ software v2.1.

RESULTS AND DISCUSSION

The clinical signs presented by the calf in this report are characteristic of infections by *A. marginale* and similar to those observed by Calderon Gonçalves et al., 2007), in a newborn calf of the girolando breed.

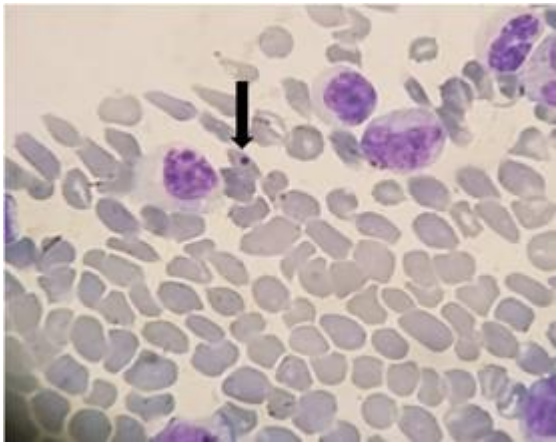
The total red blood cell count was determined to be 5.53 x 10^6/µL. Hypochromic macrocytic anemia, leukocytosis and lymphocytosis with regenerative left deviation, hypoproteinemia (5.60 g/dL) with hypoalbuminemia (2.40 g/dL), decreased creatinine (0.14 mg/dL), GGT elevation (371.0 U/L) and respiratory acidosis were evidenced (Table 1). Proceeding to smear calf blood we observed intracellular inclusions suggestive of *Anaplasma marginale* (Figure 1).

Table 1 – Hematological and biochemical profile of the calf diagnosed with *Anaplasma marginale*

Erythrogram	**Results**	**Reference values**
Red blood cells	5,53 x10^6/mm^3	6,63-8,81
Hemoglobin	8,0 g%	8,69 – 12,29
Hematocrit	29,0 %	26 – 38
Plasm protein	6,0 g/dL	5,7 – 7,1
ACV[1]	52,44 fl	38,8 – 43,4
MCHC[2]	27,59 g/dL	31,6 – 33,6
MHC[3]	14,47 pg	10,2 – 14,6
Fibrinogen	200 mg/dL	219 – 451
Metarrubricites	08/100	
RDW[4]	18,9 %	5 – 20
Platelets	408.000/mm^3	100.000 – 800.000
MVP[5]	6,5 fl	
Leukogram		
Total leukocytes	14600/mm^3	5810 – 9710
Myelocytes	146,00/mm^3	
Metamyelocytes	146,00/mm^3	
Rods	1460,00/mm^3	0 – 600
Segmented	4672,00/mm^3	2070 – 6150
Lymphocytes	7884,00/mm^3	1970 – 3730
Monocytes	292,00/mm^3	70 – 630
Biochemical paramaters		
Urea	32,0 mg/dL	12,0 – 65,0
Creatinine	0,14 mg/dL	0,8 – 1,4
ALT[6]	81,0 U/L	78,0 – 132,0
AST[7]	371,0 U/L	15,0 – 39,0
Creatine kinase	73,0 U/L	35,0 – 280,0
Total protein	5,60 g/dL	6,3 – 8,9
Albumin (A)	2,40 g/dL	2,8 – 3,8
Globulin (G)	3,20 g/dL	3,0 – 3,4
A:G ratio	0,75	

[1]ACV: average corpuscular volume; [2]MCHC: mean corpuscular hemoglobin concentration; [3]MCH: mean corpuscular hemoglobin; [4]RDW: red cell distribution width; [5]MVP: mean platelet volume; [6]ALT: alanine aminotransferase; [7]AST: aspartate transaminase.

Figure 1 – Inclusion of *A. marginale* in calf red blood cells. Optical microscopy (Nikon E200) at 40x magnification



Source: Authors (2023)

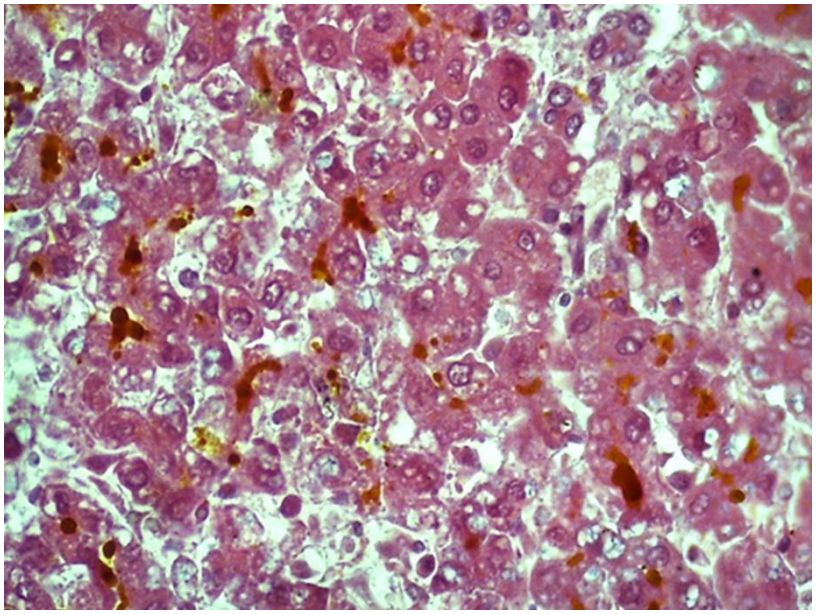
At necropsy, the animal had a regular body condition and yellowish conjunctive and subcutaneous mucosa, in addition to jaundice on subcutaneous dissection. The esophagus and abomasum were noted to be diffusely red; in the trachea large amount of pinkish-white foamy material; diffusely yellowish liver; intensely distended bladder; and slightly enlarged spleen.

Histologically, the liver showed moderate multifocal hepatocellular degeneration, predominantly in the centrilobular region, accompanied by diffuse and marked cholestasis (Figure 2). Also in the liver, discrete multifocal lymphoplasmacytic hepatitis can be observed. The spleen was moderately and diffusely congested and filled with a large number of hemosiderin-laden macrophages (hemosiderophages). In the heart, mild lymphoplasmacytic pericarditis was noted in the visceral epicardium. The lungs were diffusely and markedly congested and edematous, with moderate amounts of intravascular hematopoietic precursors in various stages of development. The brain showed accentuated multifocal neuropil vacuolization, in addition to lymphoplasmacytic and histiocytic encephalitis accompanied by multifocal and moderate malacia.

In this report, mainly neurological disorders were observed, which in cattle is not often described in *A. marginale*, but in animals with *Babesia* sp. (Bock et al., 2004), however, in blood smear the protozoan was not observed. To date, there are no descriptions of lesions in the central nervous system that have been proven to be associated with anaplasmosis in adult or neonatal cattle, nor can the lesions in the present patient be attributed to infection. One of the main causes of this lesion in particular in

newborn cattle is the transplacental infection by *Neospora caninum* (Costa et al., 2021), however, the calf's mother was serologically negative for *Neospora*.

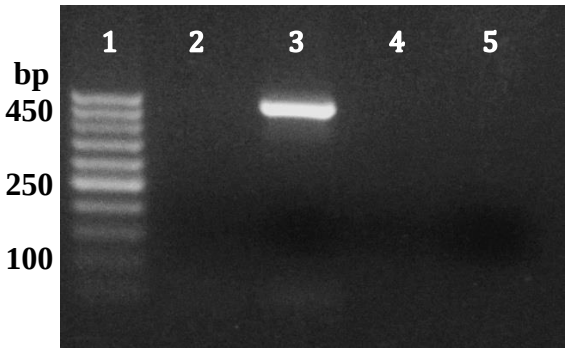
Figure 2 – Transplacental anaplasmosis in a cow. Photomicrograph of liver demonstrating diffuse, moderate cholestasis, characterized by reddish-orange material between hepatocytes, and moderate, multifocal hepatocellular degeneration (HE, x400)



Source: Authors (2023)

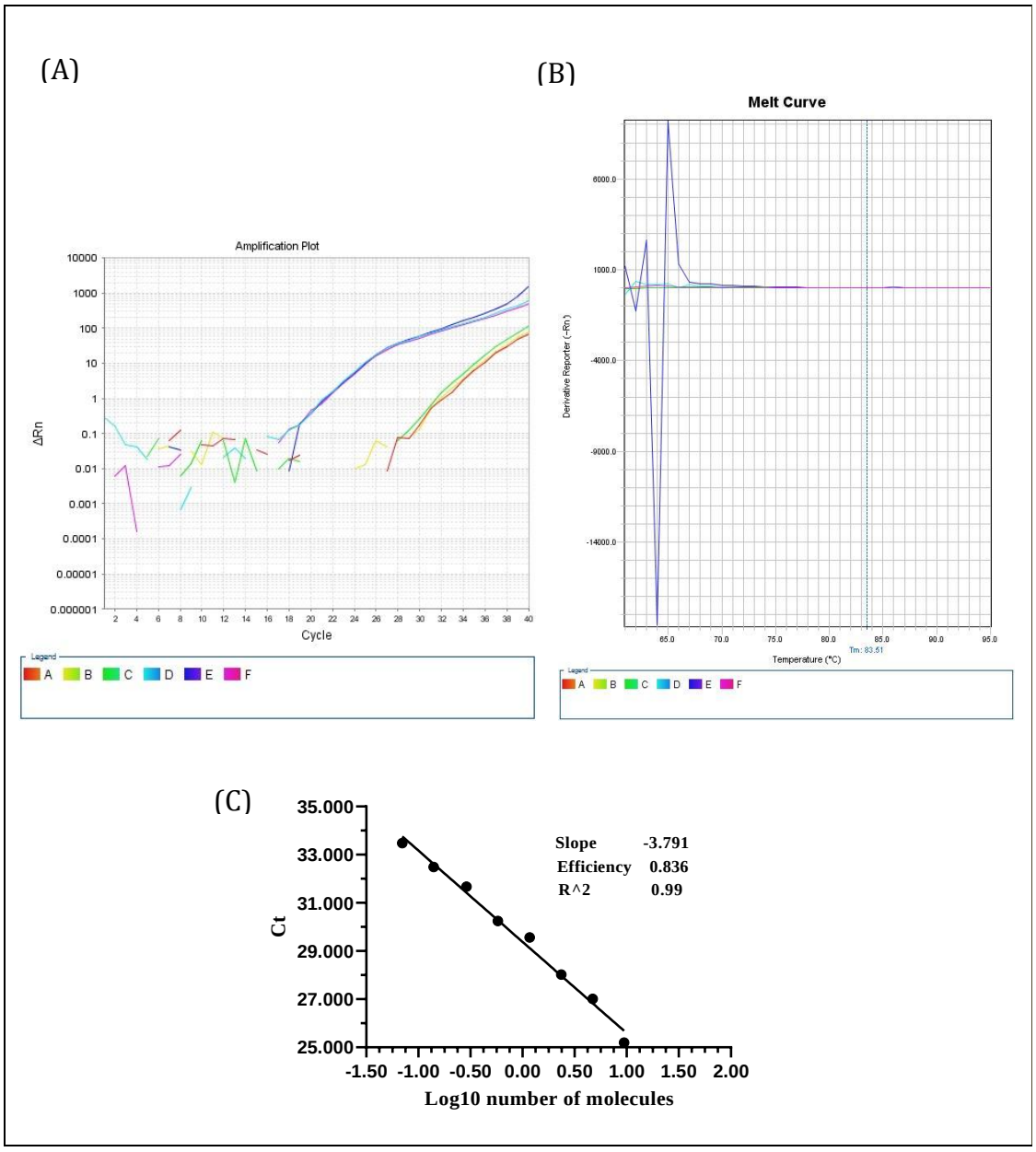
Subsequently, an *Anaplasma*-specific PCR product of 478bp was generated using ECB and ECC primers. Non-specific ECAN and HE3 primers did not generate PCR product (Figure 3). Besides, real-time-PCR detected and quantified *Anaplasma* effectively under the reaction parameter. The real-time PCR amplification curve is presented in Figure 4A. To verify the specificity of the amplified product the melt curve was obtained and showed T_m at 83.5 °C (Figure 4B). The efficiency of amplification was determined by the standard curve for ECC/ECB primers with serial dilutions DNA and calculated with the formula $E = (10^{(1/-\text{slope})} - 1) \times 100$ (Figure 4C). qPCR results showed that 3.3% of the erythrocytes in the calf's blood sample were infected. Comparatively, by counting red blood cells suggestive of infection by *A. marginale* in a blood smear under a microscope, this percentage was detected at 1.7%, a number approximately two times lower than that determined by the molecular method. This shows a higher sensitivity of pathogen detection by real-time PCR.

Figure 3 – Agarose gel electrophoresis of PCR product *Anaplasma marginale* gene. (1) 50 bp DNA Leader (Cellco Biotech); (2) Negative control: all reaction components, except template DNA; (3) PCR product from template DNA and specific primers for *A. marginale* gene; (4-5) Negative controls: non-specific primers



Source: Authors (2023)

Figure 4 – (A) The real-time amplification of *Anaplasma marginale* gene from bovine blood using StepOne™ Real-Time PCR System Thermal Cycling Block and (B) the post-amplification acquisition of melting curve. The melt curve was acquired after amplification and showed a melting temperature of 83.5 °C. (C) Sensitivity of the real-time PCR assay for detection and quantification of *Anaplasma marginale.* The standard curve was obtained by real-time PCR using 2-fold serial dilutions of bovine genomic DNA



Source: Authors (2023)

Transplacental transmission has also been reported previously in Brazil by original articles (Silva et al., 2016, 2015) on genetic diversity and molecular phylogeny of *A. marginale*. Among other techniques, the authors applied real-time PCR using primers to amplify the gene encoding Major Surface Protein 1a (MSP1a) to detect *A. marginale* in calf blood samples. Here, we have reported a case of transplacental

transmission of a calf that was born with anemia, jaundice, and neurological problems, and the blood smear showed intraerythrocytic inclusion of *A. marginale*, which was confirmed by molecular method (PCR and qPCR) target a 16S ribosomal gene.

CONCLUSIONS

In the present report, we presented a case of newborn calf with clinical symptoms suspicious of anaplasmosis and blood smear with typical *A. marginale* morula. In addition, we have applied PCR and real-time PCR assay based on the conserved fragment of RNA ribosomal gene and detected and quantified this pathogen infection in calf blood. Accordingly, we report a case of *A. marginale* transplacental transmission in this calf that died 3 days after birth. Despite being little reported, cases like the present calf occur frequently in dairy cattle, thus our study will contribute to confirming the occurrence of *A. marginale* transplacental transmission and draw attention to the epidemiological importance of bovine congenital anaplasmosis in Brazil.

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