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Toxoplasmosis and *Sarcocystis* spp. infection in wild pinnipeds of the Brazilian coast

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ABSTRACT: The protozoans *Toxoplasma gondii* and *Sarcocystis* spp. (Sarcocystidae: Apicomplexa) affect a wide variety of vertebrates. Both have been reported to infect pinnipeds, with impacts on health ranging from inapparent to fulminant disease and death. However, little is known regarding their infections and associated pathology in South American pinnipeds. We used histological techniques to survey for the presence of *T. gondii* and *Sarcocystis* spp. in 51 stranded pinnipeds from Brazil. Immunohistochemical and molecular assays were employed in those cases consistent with Sarcocystidae infection. *T. gondii* cysts were detected in the central nervous system and heart of a South American fur seal *Arctocephalus australis*, associated with meningoencephalitis, myocarditis and endocarditis, and confirmed by immunohistochemistry. Additionally, this animal presented *Sarcocystis* sp. cysts in brain and heart tissues. Four additional specimens — 2 Subantarctic fur seals *A. tropicalis*, an Antarctic fur seal *A. gazella* and another South American fur seal — presented intrasarcoplasmic cysts compatible with *Sarcocystis* spp. in muscle samples. There was no inflammation associated with the *Sarcocystis* spp. tissue cysts and all cysts were negative for *S. neurona* immunohistochemistry. The B1 gene of *T. gondii* was amplified in the 5 pinnipeds infected by Sarcocystidae protozoans. To our knowledge, this is the first report of toxoplasmosis in wild South American pinnipeds and of *Sarcocystis* spp. in South American fur seals. Detection of terrestrial parasites in aquatic mammals could be an indicator of their presence in the marine environment.

KEY WORDS: *Toxoplasma gondii* · *Sarcocystis* sp. · Mortality · Pathology · Fur seal · *Arctocephalus* · Marine mammal

1. INTRODUCTION

Marine mammal infection and associated mortality caused by Apicomplexan protozoans of terrestrial origin such as *Toxoplasma gondii* and *Sarcocystis* spp. has been reported worldwide in pinnipeds, sea otters, polar bears, cetaceans and sirenians (Buergelt & Bonde 1983, Garner et al. 1997, Conrad et al. 2005, Miller et al. 2009, Gonzales-Viera et al. 2013). *T. gondii* and *Sarcocystis* spp. have a heteroxenous

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(predator–prey) life cycle (Dubey et al. 1989, Dubey 2010). The infectious forms of these parasites are released into the environment by their definitive hosts via feces; i.e. oocysts of *T. gondii* by felids, and sporocysts of *Sarcocystis* spp. by a variety of animals according to the protozoa species (e.g. opossums for *S. neurona*; canids for *S. cruzi*) (Dubey et al. 1989, 2001, Dubey 2010). Marine mammals become infected through the ingestion of contaminated water or infected prey (Lindsay et al. 2000, Massie et al. 2010) or by vertical transmission, as observed in dolphins, pinnipeds and sea otters (Jardine & Dubey 2002, Barbosa et al. 2015, Carlson-Bremer et al. 2015, Barbieri et al. 2016, Shapiro et al. 2016).

Fatal protozoal encephalitis is one of the most serious consequences of *T. gondii* infections in marine mammals, and is also observed with some *Sarcocystis* infections (i.e. *S. neurona*) (Dubey et al. 2004, Carlson-Bremer et al. 2015). *T. gondii*-associated necrotizing and inflammatory lesions (e.g. myocarditis, myositis, lymphadenitis) and abortions have been observed in several pinnipeds (Honnold et al. 2005, Carlson-Bremer et al. 2015). *S. neurona* has also been linked to myositis in California sea lions *Zalophus californianus* (Carlson-Bremer et al. 2012), and *S. canis* reportedly caused hepatitis in Hawaiian monk seals *Neomonachus schauinslandi*, as well as California and Steller sea lions *Eumetopias jubatus* (Dubey et al. 2003, Yantis et al. 2003, Welsh et al. 2014). *Sarcocystis* sp. also caused acute hepatic necrosis in California sea lions (Mense et al. 1992). Nevertheless, asymptomatic *T. gondii* infections have also been reported (Barbieri et al. 2016), and *Sarcocystis* spp. infections can be associated with non-inflammatory cysts in skeletal muscle tissue of pinnipeds (Brown et al. 1974, Mense et al. 1992).

In this study we report *Sarcocystis* spp. infection in 5 fur seals and coinfection with *T. gondii* causing clinical disease in one animal.

2. MATERIALS AND METHODS

2.1. Sample collection

We evaluated 51 pinnipeds of the family Otariidae: 37 South American fur seals *Arctocephalus australis*, 13 Subantarctic fur seals *A. tropicalis*, and 1 Antarctic fur seal *A. gazella*. All animals were found by marine mammal rehabilitation centers between 2000 and 2014, during passive monitoring along the southern and southeastern Brazilian coast (states of Rio de Janeiro, São Paulo, Santa Catarina and Rio Grande do Sul; Fig. 1). These pinnipeds had either stranded dead, or died after stranding or during rehabilitation. All tissue samples were collected during necropsies (Geraci & Lounsbury 2005), fixed in 10% formalin or frozen at −20/−80°C and stored at the Marine Mammal Tissue Bank of the Laboratory of Wildlife Comparative Pathology, School of Veterinary Medicine and Animal Science, University of São Paulo, Brazil.

2.2. Histopathological examination

Histological evaluation was performed on available formalin-fixed tissue samples embedded in paraffin, sectioned 5 µm thick and stained with hematoxylin and eosin (H&E). Additionally, when deemed necessary, Periodic acid–Schiff, Masson's trichrome and Congo red stains were used, respectively, to characterize tissue cysts, fibrosis and amyloidosis.

2.3. Immunohistochemistry

Deparaffinized 3–4 µm sections of formalin-fixed paraffin-embedded (FFPE) tissues that contained *Toxoplasma gondii*-like cysts upon histopathological examination were stained immunohistochemically with polyclonal goat antibody against *T. gondii* (VMRD), followed by biotinylated polyclonal anti-goat secondary antibody produced in rabbit (Di Guardo et al. 2010), using 1:400 and 1:600 dilutions of the primary and secondary antibodies, respectively. All tissue samples presenting cysts were evaluated by immuno-

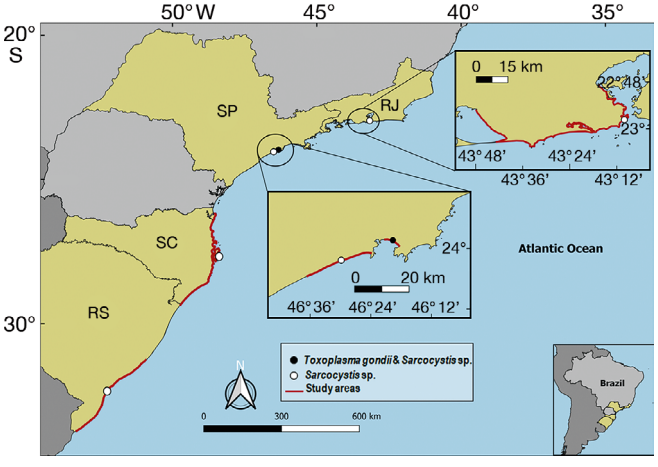

Fig. 1. Origin of the studied pinnipeds from Brazil found between 2000 and 2014 (red lines), including the cases positive for *Sarcocystis* sp. and *Toxoplasma gondii* (black dot) or *Sarcocystis* sp. (white dots)

histochemistry (IHC) for *Sarcocystis neurona* at the Animal Parasitic Diseases Laboratory–Agricultural Research Service–United States Department of Agriculture (APDL-ARS-USDA), as described by Hamir et al. (1993).

2.4. Molecular assay

DNA extraction was performed using ReliaPrep™ FFPE gDNA Miniprep System (Promega), according to the manufacturer's instructions, in two 5 µm thick sections from each FFPE tissue sample that contained Sarcocystidae-like cysts. Subsequently, PCR analysis was used to amplify a 115 bp sequence from a specific repetitive region of the B1 gene of *T. gondii* using primers B22-B23, adapted from Mesquita et al. (2010), followed by reamplification with the same pair of primers and conditions. Both PCR amplifications were made by one initial denaturation cycle at 95°C for 5 min, 40 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1 min. The procedure was completed by a final cycle extension at 72°C for 10 min. Each amplification run contained negative and positive controls. PCR-positive samples were confirmed by repeating the PCR, and by purification and direct DNA sequencing of the amplicons. The obtained sequences were aligned using the ClustalW algorithm in Mega7 software (Kumar et al. 2016), and compared with those available in GenBank database using online BLASTn search (www.ncbi.nlm.nih.gov/BLAST). Subsequently, our sequences and those closest from GenBank were aligned using the ClustalW algorithm, and the nucleotide percentage of identity was calculated based on the *p*-distance (*p*-distance × 100) using Mega7 (Kumar et al. 2016).

3. RESULTS

3.1. Gross and histopathological findings

Microscopically, 5 of 51 (9.8%) animals presented tissue cysts histologically compatible with *Sarcocystis* spp.: 2 South American fur seals, 2 Subantarctic fur seals and 1 Antarctic fur seal (Cases 1–5; Table 1, Fig. 2). *Toxoplasma gondii*-compatible cysts were observed in 1 of 51 (2%) pinnipeds, a South American fur seal. Gross descriptions were available for Cases 3, 4 and 5 (Table 1). The results of histopathological examination, as well as the Apicomplexa species observed in these animals, are summarized in Table 1.

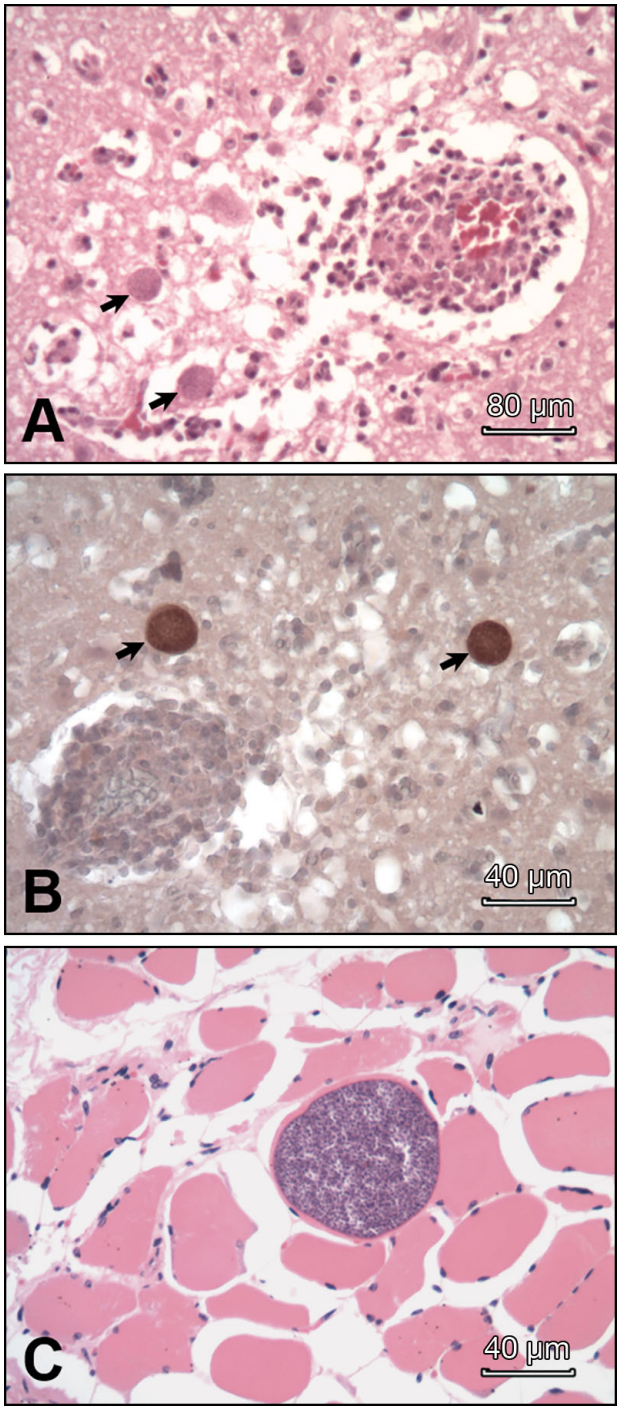

Fig. 2. Microscopic lesions and immunohistochemical findings in Sarcocystidae-positive animals. (A) Case 2: South American fur seal *Arctocephalus australis* brain. Mild mononuclear meningoencephalitis with presence of protozoan tissue cysts (arrow). H&E; scale bar = 80 µm. (B) Case 2: South American fur seal brain. *Toxoplasma gondii*-positive tissue cysts (arrow), stained immunohistochemically in brown. Scale bar = 40 µm. (C) Case 3: Subantarctic fur seal *A. tropicalis* skeletal muscle. *Sarcocystis* sp. tissue cyst containing numerous bradyzoites. H&E; scale bar = 40 µm

Table 1. Individual data and pathological findings of the pinnipeds infected by *Sarcocystis* sp. and/or *Toxoplasma gondii*. M: male; F: female; A: adult; J: juvenile; ni: no information. States in which strandings occurred: RJ: Rio de Janeiro; SP: São Paulo; SC: Santa Catarina; RS: Rio Grande do Sul

Animal ID	Species	Sex, age	Size, weight	Stranding date (dd-mm-yy) and location/history (H)	Gross (G) and histopathological findings	*T. gondii*	*Sarcocystis* sp.
MM180 (Case 1)	Subantarctic fur seal	ni	ni	21-07-02 Rio de Janeiro (RJ)[a]	**Oropharynx:** *Sarcocystis* sp. intrasarcoplasmic cyst[c,d]	–	+
					Trachea: mild to moderate diffuse lymphoplasmacytic tracheitis	–	–
					Lung: multifocal to coalescent parasitic pyogranulomatous pneumonia	–	–
					Stomach: ulcerative gastritis, multifocal hemorrhage	–	–
					Small intestine: mild diffuse lymphocytic enteritis	–	–
					Large intestine: *Dyphillobotrium* sp. associated enteritis	–	–
					Kidney: focal mononuclear nephritis, primary glomerulocystic	–	–
					Liver: mild congestion, mild to moderate cellular edema, macro and microgoticular degeneration	–	–
					Spleen: lymphoid hyperplasia	–	–
MM201 (Case 2)	South American fur seal	F	ni	1-11-00 Santos (SP) (23.96° S, 46.33° W)	**Brain:** mild multifocal mononuclear meningoencephalitis and cortical perivascular mononuclear cuffing associated with *Toxoplasma gondii* cysts; cysts of *Sarcocystis* sp.[c,d]	+	+
					Heart: mild mononuclear endocarditis and mild to moderate multifocal to coalescent perivascular mononuclear myocarditis associated with *T. gondii* cysts; *Sarcocystis* sp. intrasarcoplasmic cyst in myocardium[c,d]	+	+
					Lung: mild focal mononuclear parasitic pneumonia, moderate pulmonary edema	–	–
					Lymph node: moderate lymphoid hyperplasia	–	–
					Liver: mild to moderate periportal chronic hepatitis, multifocal necrosis	–	–
					Kidney: mild to moderate multifocal chronic interstitial nephritis	–	–
					Spleen: moderate diffuse granulocytic splenitis	–	–
					Stomach: mild multifocal mononuclear gastritis in mucosa and submucosa	–	–
					Colon: mild multifocal mononuclear colitis	–	–
MM209 (Case 3)	Subantarctic fur seal	M, A	164.5 cm 60 kg	6-09-01 Praia Grande (SP) (24.00° S, 46.41° W) H: found dead, cachectic	**G:** presence of one broken tooth, parasitic cysts in subcutaneous tissue, pulmonary edema, emphysema and congestion, heart valve fibrosis, stomach ulcers, hemorrhagic enteritis, mediastinal lymphadenomegaly, adrenal congestion, nematode and acanthocephalan parasites in intestines and nematodes in stomach		
					Skeletal muscle: *Sarcocystis* sp. cyst[c,d]	–	+
					Diaphragm: *Sarcocystis* sp. cyst[c]		+
					Heart: moderate multifocal fibrosis and thrombus	–	–
					Lung: acute, severe, fibrinohaemorragic bacterial pneumonia	–	–
					Lymph node: moderate multifocal to diffuse lymphadenitis	–	–
					Liver: mild to moderate lymphocytosis	–	–
MM607 (Case 4[b])	Antarctic fur seal	F, A	123 cm 28.7 kg	27-06-12 Rio Grande (RS) (32.19° S, 52.16° W) H: stranded alive in poor body condition. The animal was found dead the next day	**G:** several dog bites, Mallophaga ectoparasites, hematomas in neck and pectoral fin, pulmonary congestion, emphysema, multifocal light-tanned granulomatous lesions by *Parafilaroides* sp., light-tanned multifocal areas in all the hepatic lobes, gastritis, low volume of gastric content, *Contracaecum* sp., *Corynosoma* sp. in the intestines, and severe large intestine enteritis		
					Skeletal muscle: *Sarcocystis* sp. intrasarcoplasmic cyst[c,d]	–	+
					Lung: moderate to severe congestion, mild to moderate diffuse mononuclear interstitial parasitic pneumonia (*Parafilaroides* sp.) and multifocal to coalescent granulocytic bronchopneumonia	–	–
					Lymph node and spleen: lymphocytolysis and follicular mantle hypoplasia	–	–
MM620 (Case 5)	South American fur seal	M, J	100 cm	23-08-14 Florianópolis (SC) (27.71° S, 48.50° W) H: stranded alive in poor body condition, unable to eat. Despite treatment, the animal had progressive weight loss and died 10 d later	**G:** parasitic subcutaneous cyst, pulmonary, tracheal and mesenteric vessel congestion, pale spleen and intestinal nematodes		
					Skeletal muscle: *Sarcocystis* sp. intrasarcoplasmic cysts[c,d]	–	+
					Trachea: mild to moderate diffuse mononuclear tracheitis	–	–
					Lung: mild congestion, mild to moderate diffuse mononuclear interstitial parasitic pneumonia (*Parafilaroides* sp.)	–	–
					Heart: mild to moderate congestion, mild diffuse mononuclear myocarditis	–	–
					Small intestine: mild to moderate diffuse mononuclear enteritis	–	–
					Liver: mild diffuse mononuclear hepatitis	–	–
					Lymph node: follicular hyperplasia	–	–

[a]No geographical coordinates available; [b]The microscopic findings of this case were partially described before along with PCR confirmation of *Parafilaroides* sp., *Contracaecum* sp. and *Corynosoma* sp. (Jacobus et al. 2016); [c]No associated inflammatory response was verified; [d]Tissue PCR-positive for *T. gondii*

3.2. IHC

Tissue cysts in brain and heart tissues of Case no. 2 (South American fur seal) had detectable *T. gondii* antigen. All analyzed tissue samples from the 5 pinnipeds that presented *Sarcocystis* spp. cysts were negative for *S. neurona* IHC.

3.3. Molecular findings

Five 64 bp sequences (excluding primers) of the B1 gene (5′-TAC AAA CTG CTA AAC GGT CCG GGT GAA ACA ATA GAG AGT ACT AGA ACG TCG CCG CTA CTG CCC A-3′) were obtained from tissues taken from the 5 animals that presented Sarcocystidae cysts (oropharynx [MM180], brain and heart [MM201], and skeletal muscle [MM209, MM607 and MM620]). All sequences were identical and presented high nucleotide identities to 2 *T. gondii* sequences available in GenBank (100 and 98.4% identities to the sequences in GenBank accession nos. LN714499.1 and AF179871.1, respectively).

4. DISCUSSION

The current study describes *Sarcocystis* spp. infection in 5 pinnipeds, and toxoplasmosis in a South American fur seal (Case no. 2) with mononuclear

meningoencephalitis, myocarditis and endocarditis. Although the former animal also presented sarcocysts in the brain and heart, the inflammatory response in these tissues was associated with *Toxoplasma gondii* rather than *Sarcocystis* sp., because aside from the perivascular mononuclear cuffing, the inflammatory cells were only observed adjacent to *T. gondii* cysts. Toxoplasmosis potentially contributed to the animal's stranding, but its intensity was not enough to be considered the primary cause of death. The meningoencephalitis observed in Case no. 2 was caused by *T. gondii*, presenting *T. gondii* cysts adjacent to inflammatory lesions and without evident tachyzoites, as described in a case of toxoplasmic meningoencephalitis in a northern elephant seal *Mirounga angustirostris* (Dubey et al. 2004) and in some *T. gondii*-positive California sea lions *Zalophus californianus* and Hawaiian monk seals *Neomonachus schauinslandi* with encephalitis and/or meningoencephalitis, respectively reported by Carlson-Bremer et al. (2015) and Barbieri et al. (2016). Mixed *T. gondii* and *Sarcocystis* sp. infection has been previously described in Hawaiian monk seals and New Zealand fur seals *Arctocephalus forsteri* (Donahoe et al. 2014, Barbieri et al. 2016). Polyparasitic infections have been suggested as an important factor regarding disease severity in marine mammals (Gibson et al. 2011); however, additional research is necessary to clarify the potential synergic effects of this condition. Additionally, Case no. 2 presented moderate myocarditis (mainly perivascular) and mild endocarditis, with the presence of *T. gondii* adjacent to the inflammatory infiltrate, and noninflammatory *Sarcocystis* sp. cysts. Therefore, the observed lesions were associated with *T. gondii*. Toxoplasmic myocarditis has been described in otariids, including a northern fur seal *Callorhinus ursinus*, a New Zealand fur seal and California sea lions (Holshuh et al. 1985, Donahoe et al. 2014, Carlson-Bremer et al. 2015).

This is the first molecular confirmation and immunohistochemical and histological detection of *T. gondii* in a South American pinniped, although antibodies against *T. gondii* have been previously detected in South American sea lions from Chile (Sepúlveda et al. 2015). Our findings confirm the contamination of the regional marine environment by this terrestrial agent, as previously observed in cetaceans and oysters (Esmerini et al. 2010, Gonzales-Viera et al. 2013). *T. gondii* had been previously detected in other species of Southern Hemisphere pinnipeds by histology, IHC and PCR, including a New Zealand sea lion *Phocarctos hookeri* and a New Zealand fur seal (Donahoe et al. 2014, Roe et al. 2016).

Five pinnipeds from this report presented *Sarcocystis*-like tissue cysts; however, due to the low resolution of histopathology, this is likely the minimum number of infections. The observed histopathological findings — intramuscular *Sarcocystis*-like cysts in the absence of myositis — were in agreement with previous reports of skeletal and cardiac muscle samples of pinnipeds (Brown et al. 1974, Mense et al. 1992), resembling the noninflammatory muscular tissue cysts observed in the majority of *Sarcocystis* spp. infections in intermediate hosts (Cooper & Valentine 2015). To the authors' knowledge, this is the third report of *Sarcocystis* sp. infections in pinnipeds from South America, with previous description of cysts consistent with *Sarcocystis* sp. in the skeletal muscle of a South American sea lion *Otaria byronia* from Chile (Sepúlveda et al. 2015) and a Subantarctic fur seal from Brazil (Reisfeld et al. 2019). Our findings also constitute the first report of *Sarcocystis* sp. infection in a South American fur seal. Regarding Antarctic fur seals, *Sarcocystis* sp. has been previously identified in edematous muscle of a specimen from the South Georgia Archipelago (Antarctic) (Baker & Doidge 1984).

Although an identical *T. gondii* sequence was detected in 5 different pinnipeds, only one South American fur seal presented *T. gondii*-characteristic lesions, while the other 4 animals were asymptomatic. It is important to highlight that, despite the large number of *T. gondii* reports worldwide, only 3 sequences matched our amplified sequence after a BLASTn search — 2 of them with at least the same length as our sequences when compared — thus, further research to characterize the genotype of this strain infecting South American pinnipeds is warranted.

Herein we detected novel cases of *T. gondii* and *Sarcocystis* spp. infection in pinnipeds from Brazil, including the first report of toxoplasmosis in a South American pinniped. All the *Sarcocystis* spp. tissue cysts were negative for *S. neurona* by IHC. Our findings indicate the contamination of the ocean by terrestrial agents, and warrant further investigations to elucidate *T. gondii* strains and *Sarcocystis* species infecting South American pinnipeds, their associated pathology and potential impact on these populations, and the potential sanitary implications regarding the detection of the zoonotic agent *T. gondii* in the marine environment.

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