Microbial Pathogenesis 160 (2021) 105186

Contents lists available at ScienceDirect

Microbial Pathogenesis

journal homepage: www.elsevier.com/locate/micpath

Cellulitis-related *Rhodococcus equi* in a cat harboring VAPA-type plasmid pattern

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ARTICLE INFO

Keywords:
Feline rhodococcosis
Extrapulmonary signs
Virulence plasmid profile
pVAPA-type
Pathogenicity
MALDI-TOF MS

ABSTRACT

Rhodococcus equi is a well-known intracellular facultative bacterium that is opportunistic in nature, and a contagious disease-causing agent of pyogranulomatous infections in humans and multihost animals. Feline rhodococcosis is an uncommon or unnoticed clinical condition, in which the organism is usually refractory to conventional antimicrobial therapy. The pathogenicity of the agent is intimately associated with plasmid-governed infectivity, which is attributed to the presence of plasmid-encoded virulence-associated proteins (Vap). Three host-adapted virulence plasmid types (VAPs) have been distinguished to date: pVAPA, pVAPB, and pVAPN, whose infections are related to equine, pig, and bovine or caprine origin, respectively, while humans are infected by all three VAP types. Most virulence studies with *R. equi* plasmid types in animals involve livestock species. Conversely, data on the pathogenicity and human relevance of the virulence plasmid profile of *R. equi* isolated from cats remains unclear. This report describes a case of cellulitis-related *R. equi* that harbors the pVAPA-type in a cat with cutaneous lesion. Long-term therapy of the cat using marbofloxacin, a broad-spectrum third-generation fluoroquinolone, resulted effectiveness. pVAPA is a host-adapted virulent type that has been associated predominantly with pulmonary foal infections. Our cat had a history of contact with other cats, livestock (including horses), and farm environment that could have favored the transmission of the pathogen. Besides no clear evidence of cat-to-humans transmission of the pathogen, the identification of *R. equi* harboring pVAPA-type in a cat with cutaneous abscessed lesion represent relevance in human health because this virulent type has been described in people worldwide with clinical rhodococcal disorders.

1. Introduction

Rhodococcus equi is a gram-positive intracellular facultative, nonmotile, nonspore-forming bacterium. The pathogen is opportunistic in nature, causing different pyogranulomatous clinical disorders in humans, multihost domestic species, and wildlife [1–3]. It is a well-known soilborne microorganism distributed globally and widely found on the surface of soil and manure, particularly in horse-breeding farms [1]. The bacterium is eliminated through the feces of domestic animals, especially livestock species such as horses [4], cattle [5], and pigs [6], and less commonly from companion animals. Currently, *R. equi* was identified for the first time in the feces of nondiarrheic cats that

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https://doi.org/10.1016/j.micpath.2021.105186
Received 21 April 2021; Received in revised form 9 July 2021; Accepted 6 September 2021
Available online 9 September 2021

inhabited horse farm environment [7].

The pathogenicity of *R. equi* is intimately related to its intracellular lifestyle, which enables the pathogen to invade, survive, and replicate inside phagocytic cells and to evade of the immune response mechanisms of the host [2,8], although the major virulence factor of the pathogen has been attributed to the presence of large virulence-associated plasmids that carry the *vap* pathogenicity island [9], which encode a number of distinct proteins (VAPs) linked to virulence [8].

Three host-adapted virulence plasmid types have been distinguished to date and described based on a novel unified nomenclature (pVAP): pVAPA (formerly VapA or virulent), pVAPB (formerly VapB or intermediately virulent), and pVAPN (N for either no-A or no-B) [1,8]. pVAPA type is harbored by *R. equi* isolated from foals, and it is predominantly related to typical life-threatening suppurative pneumonia of young horses up to six months of age, which is known as equine-type [1,4]. The pVAPB type has been predominantly found in strains from pigs and wild boars (porcine type) isolated from the lymph nodes with and without lesions [10,11]. pVAPN type [12], a bovine- or caprine-associated plasmid-type, has been identified from cattle (bovine-type) and caprine species, and in particular, it has been recovered from the lymph nodes of slaughtered cattle [5], as well as pulmonary and extrapulmonary disorders of goats [13]. Contrasting with evidence for the apparent selectivity of animal species, the three host-adapted plasmid types are able to infect humans [8], particularly patients living with human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) [14,15]. Isolates lacking the three known virulent types, so-called plasmidless or "avirulent" isolates, have been found in the feces of livestock species, and the environments of breeding farms, parks, and yards, and they occasionally infect humans and domestic animals [1,8,14,15], although strains from pig or bovine origin have been identified more frequently than equine type in human rhodococcosis [1,5,14].

Clinical rhodococcosis is a major disease of veterinary medicine in foals up to 6 months of age that develop pyogranulomatous bronchopneumonia with remarkable abscessation, and less commonly enteric, articular, and ocular signs [2,4]. In turn, there is a paucity of data regarding feline *R. equi* infections [16], and clinical cases may be considered rare [17,18]. In addition, data on the pathogenicity and human health relevance of the virulence plasmid profile of *R. equi* strains isolated from cats is scarce or neglected [18,19].

Inhalation from soil particles, ingestion of contaminated food, traumatic percutaneous injury, contamination of mucous membranes or previous lesions, and tissue inoculation secondary to bites or scratches during fights are possible routes of transmission of the pathogen to felines [17,18].

Most feline *R. equi*-induced infections cause pneumonia [16,20]. Less commonly, cutaneous purulent lesions [18], fistulas, or multiple sinuses predominantly in the extremities [16], cellulitis and enlargement of regional lymph nodes [21], and mesenteric and mediastinal lymphadenitis have also been reported [22]. Systemic or disseminated forms have also seen as hepatitis, splenomegaly, pyothorax, myositis, and osteomyelitis [16,23], and are commonly related to underlying conditions or secondary to immunosuppressive viral infections [17].

A number of classes of antimicrobials and their combinations have been used to treat feline rhodococcosis [16,17,24], although no standard therapy approach is recommended. Variable outcomes have been observed among different antimicrobial therapy of feline rhodococcosis [16,18,21,23], although cats with systemic or disseminated forms respond poorly to treatment [17].

In this scenario, we report clinical and epidemiological diagnostic features, microbiological examination, and antimicrobial therapy of cellulitis-related *R. equi* in a cat. The mass spectrometry diagnostic method enables confirmation of etiological agent. The isolate carried a VAPA plasmid-type pattern that has been predominantly detected in foal pneumonia.

2. Materials and methods

This study was conducted under the Ethics Committee on Animal Use (CEUA) guidelines of the School of Veterinary Medicine and Animal Sciences, São Paulo University-UNESP, Botucatu, SP, Brazil (protocol number 169/2014). An adult Persian cat with a 2-week history of an inguinal cutaneous lesion was admitted to a Veterinary Hospital in the state of Ceará, northwest Brazil. According to the owner, the cat had been recently adopted, and no previous history of vaccination status was available. The cat lived in an environment with contact with other cats and livestock species, including horses and cattle. On arrival at Veterinary Hospital, the cat showed fearful behavior and a painful reaction upon initial palpation of the inguinal region.

In the clinical examination, a nodular, erythematous, firm, painful, nonpruritic, fistulized lesion associated with purulent blood exudation and lymphadenomegaly in the inguinal region, with extending edema up to the penis, was observed (Fig. 1A). The cat was subjected to hematological and serum biochemical tests, as well as a thoracic image examination. No other clinical signs were observed. In addition, blood samples were subjected to the diagnosis of feline leukemia virus (FeLV) and immunodeficiency virus (FIV) using a commercial qPCR kit (Alere Fiv Ac/FeLV Ag, Alere®, São Paulo, Brazil). Initially, the cat received three applications of benzylpenicillin (24,000 UI/kg every 4 days) by a referred veterinarian without clinical success.

Purulent material from the lesion was submitted for Gram and Diff-Quick cytology staining. Biopsies from lesions were submitted for histological and microbiological culture in sheep blood agar medium (5%) and MacConkey agar medium (Oxoid®, São Paulo, Brazil) and aerobically incubated for 3 days at 37 °C. Material from biopsies was also cultured in Sabouraud agar medium (Oxoid®, São Paulo, Brazil) and aerobically incubated for 15 days at 37 °C. Furthermore, material from the lesion was cultured aerobically in Lowenstein-Jensen medium (Oxoid®, São Paulo, Brazil) for 90 days at 37 °C.

The microorganisms were initially identified based on conventional morphological and phenotypic aspects (*e.g.*, morphology to staining methods, features of colonies, CAMP test using *Staphylococcus aureus*) [25]. Bacterial confirmation at the species level was performed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS, Bruker and Daltonics™, Bremen, Germany) with a 337-nm laser. Spectra were analyzed between 2.000 and 20.000 *m/z* using FlexControl 3.3 software. Characterization of the microorganisms at the genus and species levels was considered with $\geq$1.7 and $\geq$ 2.0, respectively [26,27].

Isolates were subjected to an *in vitro* antimicrobial disk diffusion test according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [28] using nine antimicrobials from five different classes, as follows: 1) cephalosporins (ceftriaxone 30 μg, cefovecin 30 μg), 2) fluoroquinolones (levofloxacin 5 μg, marbofloxacin 5 μg), 3) macrolides (azithromycin 15 μg, clarithromycin 15 μg, erythromycin 15 μg), 4) synthetic or derivative penicillins (amoxicillin/clavulanic acid 30 μg), and 5) rifamycins (rifampin 5 μg).

Isolates compatible with *Rhodococcus equi* were subjected to PCR analysis to investigate the plasmid virulence types VAPA, VAPB, and VAPN. In brief, PCR was performed as previously reported with slight modifications [15]. Namely, *Rhodococcus equi* isolate (BRA 198) was cultured in BHI broth (Becton Dickinson, Sparks, MD) at 30 °C for 48 h with shaking. Genomic DNA from cultured *R. equi* cells treated with lysozyme from chicken egg white (Sigma-Aldrich, St. Louis, MO) was extracted using a QIAamp DNA purification *kit* (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The yield and purity of the extracted DNA were determined using a NanoDrop One^{c} spectrophotometer (Thermo Fisher Scientific, Wilmington, DE).

To evaluate the specificity of the designed primer sets for detecting *choE*, *vapA*, *vapB*, and *vapN* as presented in previous report [15], PCR was performed using Tks Gflex™ DNA Polymerase and the respective primer pairs. The amplification reactions were performed using a

Fig. 1. Purulent cutaneous lesion in a Persian cat caused by *Rhodococcus equi*: **A** = Nodular, erythematous, firm, fistulized lesion, associated with purulent blood exudation in the inguinal region, with extending edema up to the penis **B** = The treatment with marbofloxacin for 40 days, reached complete resolution of the lesion.

GeneAmp PCR System 9700 (Thermo Fisher Scientific). Thermal cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 60 s were repeated 30 times. The presence of PCR amplicons was confirmed by electrophoresis using 1% Agarose NA gels (GE Healthcare UK Ltd, Buckinghamshire, UK). The ATCC33701 strain [29] was used as a positive control for the *vapA* gene, and the OKI2013Lv1 strain [30] was used as a positive control for the *vapN* gene.

For the plasmid profiles of pVAPA, plasmid DNA was digested using the restriction endonucleases *Eco*RI as described previously [31]. Restriction fragment length polymorphism (RFLP) patterns of pVAPA harbored by each strain were confirmed by electrophoresis using 1% Agarose NA gels [32].

3. Results

The hematological examination revealed a leukocyte count of 11,2 $\times$ 10^9/L, neutrophil count of 8,5 10^9/L, and erythrocyte count of 9,9 $\times$ 10^9/L, while biochemical tests showed unremarkable values. Radiography examination revealed no significant alterations. FIV and FeLV investigation was negative. A pyogranulomatous inflammatory infiltrate with multiple intracellular coccoid-to-rod organisms was observed in the cytopathological and histopathological examination.

Microbiological culture of purulent material from the lesion revealed irregular, mucoid, nonhemolytic, coalescent, white-to-gray colonies in sheep blood agar medium after 48 h. Gram staining of the colonies

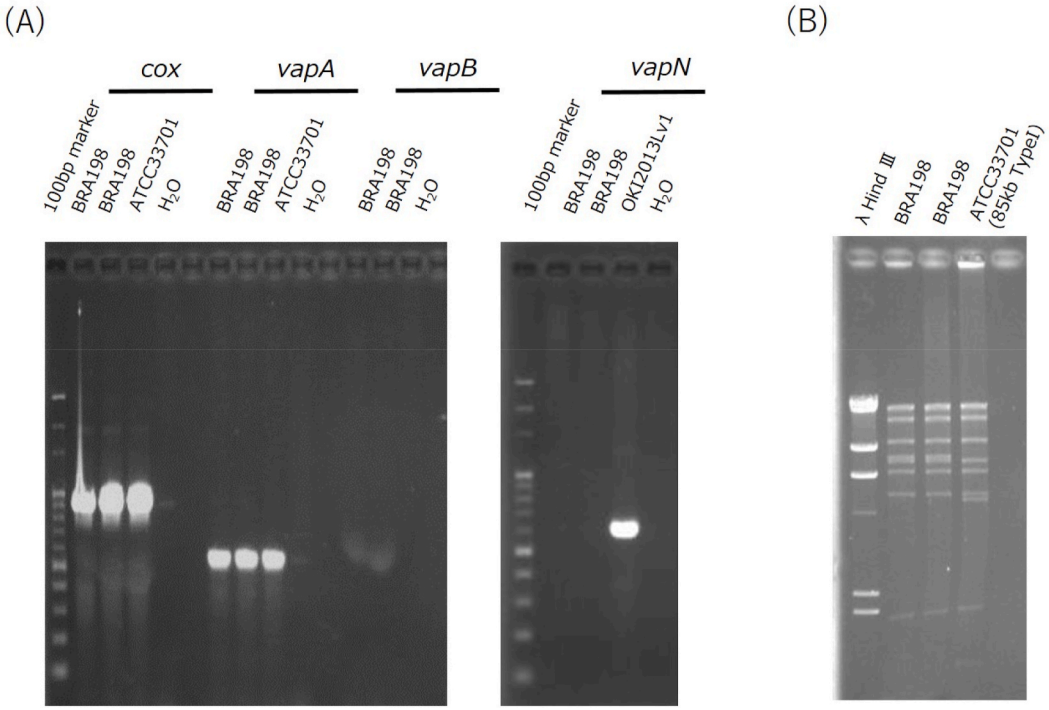

Fig. 2. Surveying of *vap* genes (A) and typing of pVAPA plasmid (B). The genomic DNA and plasmid of the *Rhodococcus equi* BRA198 strain was extracted in duplicate. Restriction endonucleases *Eco*R1 was used for plasmid typing.

revealed gram-positive coccobacillary organisms, and a positive CAMP test, compatible with *R. equi*. There was no microbial growth in the MacConkey, Sabouraud, and Lowenstein-Jensen media.

MALDI-TOF MS spectra of the isolate was >2.0, and the agent previously isolated in sheep blood agar medium was confirmed as *R. equi*. Fig. 2 shows the results of PCR and plasmid RFLP patterns harbored by *R. equi* isolate (BRA 198 strain), which carried an 87-kb type I plasmid harboring *vapA* gene (VAPA-type) [33]. This strain did not harbor any other *vap* plasmids.

In vitro antimicrobial susceptibility testing showed that the isolate was susceptible to all drugs tested, resulting in subsidized treatment with marbofloxacin (2.7 mg/kg/24 h, for 40 days), showing complete resolution of the lesion (Fig. 1B).

4. Discussion

The routine diagnosis of feline rhodococcosis has been based on epidemiological and clinical features, bacteriology, imaging, and cytological and histological examination [16–18,21]. Previous reports of cutaneous lesions caused by *R. equi* infections have observed the presence of numerous intramacrophage gram-positive coccoid-to-rod organisms and a typical pyogranulomatous reaction characterized by a great number of macrophages and neutrophilic suppurative foci [18,21], which agrees with the cyto- and histological findings observed in our cat.

In the current report, leukocyte and erythrocyte counts revealed normal values for canine species. Also, tests for renal and hepatic function showed unremarkable results, and no systemic signs or pulmonary image abnormalities were seen, indicating an infection exclusively cutaneous [17,18,21]. Even though there is no consistency of outcome based on localization of lesions or severity of clinical signs [16], the systemic or disseminated forms appear to determine a poor prognosis for feline rhodococcosis [17], a factor that may have influenced the success of therapy in the present case, whose lesion was exclusively cutaneous.

Underlying conditions and coinfections with immunosuppressive diseases are known predisposing factors to the development of rhodococcosis among companion animals [33], particularly cats infected by feline leukemia virus (FeLV) or feline immunodeficiency virus (FIV) [17]. In this regard, no systemic signs were observed in our cat, and the animal was negative for FeLV and FIV tests, which may have contributed to the satisfactory outcome.

Mass spectrometry has been increasingly applied to the diagnosis of *R. equi* infections in humans and animals. A study that involved 154 *R. equi* strains isolated from human patients living with HIV/AIDS, pneumonic foals, and from the environment revealed fast and reliable mass spectrometry results for the diagnosis of *R. equi* [34]. In this case, mass spectrometry confirmed *R. equi* identification of species level, and we believe that MALDI-TOF MS was used for the first time to diagnose *R. equi* in a cat with clinical rhodococcosis, where the virulence-plasmid pattern of the isolate has been assessed.

Pyogranulomatous bronchopneumonia is a major clinical disorder of foals and humans infected by *R. equi* [1,2,4]. Among nonequine species, particularly companion animals, besides pulmonary signs [35], extrapulmonary disorders have been observed, including weight loss, lymphadenitis, hepatitis, myositis, osteomyelitis, cutaneous and organ abscesses [17,18,20,21], which reinforce the opportunistic nature of *R. equi* [1]. A retrospective, comprehensive large-scale study, involving the clinicopathological and radiographic features of 40 cases of feline rhodococcosis revealed that 36 (90%) animals had pulmonary involvement, whereas only 4 (10%) cats showed exclusively cutaneous lesions [16]; indicating that an exclusive skin (cellulitis) lesion reported in our cat, without systemic involvement, may be seen as an uncommon finding of feline rhodococcosis.

Inhalation of soil particles in farm environments has considered as a primary transmission route of *R. equi* to humans and animals [1,4]. In the last decade, consumption of raw or undercooked meat from bovines, pigs, or wildlife animals has been proposed as a probable source of *R. equi* infections [3,5,6,10,11], particularly to humans without a history of contact with livestock or environment of farms [5]. Hence, *R. equi* has emerged as a presumable food-borne zoonotic pathogen [11]. Conversely, traumatic percutaneous injuries, environmental contamination of previous cutaneous lesions, and tissue inoculation of *R. equi* secondary to fights appear to be probable routes to the transmission of the pathogen to felines [17,18]. In this case, the cat developed a lesion exclusively cutaneous and had a history of contact with other cats, horses, cattle, and farm environment, which could have facilitated a percutaneous injury induced by other cats [17,18] or soil contamination of a skin lesion with *R. equi*, since the pathogen is widely distributed in the soil of farm environment, particularly from livestock breeding farms [1,4,36].

For decades, the combination of rifampin and macrolides, initially erythromycin and, most recently, clarithromycin and azithromycin, constitutes the main therapy for equine rhodococcosis [2,4,24], which historically represent the domestic species most frequently affected by *R. equi* [1,2,4]. Rifamycins, macrolides, aminoglycosides, sulfonamides, fluoroquinolones, and their associations represent the main groups of antimicrobials for rhodococcal therapy in livestock [2,4,24] and companion animals [17]. Nonetheless, lipid-soluble or bactericidal drugs are the first choice because they achieve therapeutic levels in suppurative foci of tissues and phagocytic cells [1,4,24], although the increase of macrolide/rifampicin resistance has become a substantial concern [2,37]. A prolonged course of therapy with marbofloxacin, a broad-spectrum third-generation synthetic fluoroquinolone developed for veterinary practice, was effective in our cat. Because *R. equi* is found in the cytoplasm of phagocytic cells [1], the efficacy of therapy in the current report could be attributed, in part, to the intracellular action of this fluoroquinolone [24,38].

The *in vitro* antimicrobial susceptibility profile of our *R. equi* isolate revealed sensitivity to marbofloxacin. In a retrospective study involving 40 cats with clinical rhodococcosis, 37 animals had a history of antimicrobial treatment, of which 14 (37.8%) received marbofloxacin. Of these, 8 (57.1%) recovered, 5 (35.7%) died, and 1 (7.2%) was euthanized. Most of the cats that recovered had *R. equi* isolates susceptible *in vitro* to marbofloxacin [16], reinforcing the importance of *in vitro* susceptibility tests previous therapy approaches [24].

The pathogenicity of *R. equi* to human and animal hosts has been intimately related to the presence of the three host-associated plasmid types, *i.e.*, pVAPA, pVAPB, and pVAPN, which have been predominantly found in equines [1,2,39], pigs [10,11,36], and bovine [5] or caprine [13] infections, respectively. Conversely, there is no specific virulence type associated with companion animal infections, because pVAPA, pVAPB, and, recently, pVAPN have been reported in cats [18] and dogs [19,33,40]. In fact, there is a paucity of data regarding the virulence plasmid patterns of *R. equi* that infects cats worldwide [18,19]. In this regard, investigation of the *vapA* and *vapB* genes among 18 *R. equi* strains isolated in nine cats and nine dogs with rhodococcosis from different countries revealed five cats and one dog carrying pVAPA [19]. A report of clinicopathological, histopathological, and microbiological features has been described in a male cat from Brazil with a cutaneous lesion caused by *R. equi* that carried pVAPA-type [18]. Likewise, our cat harbored a VAPA-type plasmid that has been typically found in pneumonic foals worldwide [1,2], including in foals from Brazil [39].

An increasingly number of *R. equi* infections in humans have been reported in the coming years [5,15,41]. Nonetheless, conversely to apparent host-driven pVAP-types related to livestock *R. equi* infections, the clinical cases of human rhodococcosis around the world have been caused by the three known types pVAPB, pVAPN, and pVAPA, particularly among patients who live with HIV/AIDS [5,14,15,41]. This evidence indicates that human infections possess a zoonotic origin and the domestic animals represent the source of infections for humans [8]. Although the real impact of companion animals as a source of *R. equi*

infections for humans remains unclear, the VAPA-type detected in our cat has been reported in human patients from different countries, *e.g.*, Brazil [42], Japan [15], the USA [43], and Cuba [41]. Thus, studies focused on the virulence-plasmid profile of *R. equi* infections among domestic animals, including cats [18,19], are important to investigate the geographical distribution of pVAP-types and the relevance of different domestic animal species in the transmission of virulent *R. equi* for humans, as well as to adopt control and preventive measures against the disease.

5. Conclusion

Overall, the present report describes microbiological, cyto- and histological, and molecular diagnosis of cellulitis-related *R. equi* infection in a cat successfully treated with long-term therapy using marbofloxacin. The isolate carried a host-adapted pVAPA-type, which has been predominantly found in foal pneumonia. Besides no specific VAP-type be related to companion animals, and no clear evidence of the route of infection was determined in our cat, studies regarding the virulence plasmid patterns of feline *R. equi*-induced infections are scarce and may contribute to evaluate the relevance of cats in the transmission of virulent isolates for other animals and humans. Also, the identification of the pathogen harboring pVAPA-type in a cat with cutaneous abscessed lesion represent relevance in human health because this virulent type has also been described in people with clinical rhodococcosis.

Funding information

We appreciate the support of National Council for Scientific and Technological Development (CNPq, protocol 310345/2020-0), Brazil, for research productivity fellowship (PQ-1D) given to Márcio Garcia Ribeiro.

Author contribution statement

Conceptualization, data analysis, methodology, investigation: B.Z.L. L. Rocha, M.R. de Farias, M.G. Ribeiro, F.S. Monti, F.V.R. Portilho: Sampling: B.Z.L.L. Rocha; Microbiological diagnosis: F. Garino Júnior, M.G. Ribeiro, F.V.R. Portilho, B.O. de Almeida, A.A.L. de Souza; Molecular diagnosis: Y. Morizini, N. Sakaizawa, Y. Suzuki, T. Kakuda, S. Takai; Writing-review & editing: all the authors.

Declaration of competing interest

The authors declare no conflict of the interest.

Acknowledgments

The authors acknowledge the financial support from National Council for Scientific and Technological Development from Brazil (CNPq, protocol 310345/2020-0) and São Paulo Research Foundation (Fapesp, Protocol 2015/20585-9).

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