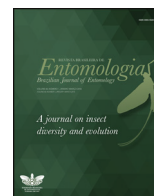










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## Molecular characterization of *Bacillus thuringiensis* strains to control *Spodoptera eridania* (Cramer) (Lepidoptera: Noctuidae) population

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### ABSTRACT

The main objective of this study was to characterize the toxicity and genetic divergence of 18 *Bacillus thuringiensis* strains in the biological control of *Spodoptera eridania*. Bacterial suspensions were added to the *S. eridania* diet. Half of the selected *B. thuringiensis* strains caused high mortality seven days after infection. The genetic divergence of *B. thuringiensis* strains was assessed based on Enterobacterial Repetitive Intergenic Consensus (ERIC) and Repetitive Extragenic Palindromic (REP) sequences, and five phylogenetic groups were formed. Despite their genetic diversity *B. thuringiensis* strains did not show any correlation between the collection sites and toxicity to larvae. Some *B. thuringiensis* strains are highly toxic to *S. eridania*, thus highlighting the potential of their endotoxins as biopesticides.

### Introduction

During the year 2017/18 the total grain yield in Brazil was estimated to be 229.53 million tons, and trends indicate further increase in the upcoming (CONAB, 2018). However, such expectation of agricultural growth may not be achieved due to the emergence of phytosanitary issues responsible for causing crop injuries in many agricultural regions. Herbivorous insects are said to be responsible for destroying one fifth of the world's total crop production annually (Sallam and Bothe, 1999).

In the past, the chemical control was the most common method employed to overcome pest spreading on crops, but over the last two decades natural approaches emerged as alternative methods to insect control. *Bacillus thuringiensis* (*B. thuringiensis*) (Berliner, 1911) is a useful tool that naturally controls insects, and it is a Gram-positive bacteria that during the sporulation stage produces protein crystalline inclusions, called Cry proteins, which have selective insecticidal activity

against different groups of insects (Yamamoto and Dean, 2000). After *B. thuringiensis* was employed as a biological control agent, the use of chemical products has progressively reduced and, as well as the environmental pollution caused by its toxic residues (James, 2015). Currently, *B. thuringiensis* sub-species represent about 98% of formulated sprayable bacterial microbial pesticides (Lacey et al., 2015). Nevertheless, it still represents only 2% of the global pesticide market (Bravo et al., 2011).

*B. thuringiensis* proteins comprise the synthesis of some endotoxins such as Crystal proteins (*cry*), Cytolytic (*cyt*) proteins, Vegetative insecticide proteins (*vip*) and thuringiensine ( $\beta$ -exotoxin) (Bravo et al., 2007). In spite of the presence of four endotoxins types, Cry proteins have been widely considered the major crystal component characterizing *B. thuringiensis* strains (Crickmore et al., 1998), mainly because of the specific toxic potential these proteins may exhibit towards insect control.

Molecular characterization of new *B. thuringiensis* genes is important due to its specific mode of action in target insects. Approximately

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770 *cry* genes were already sequenced, cataloged and qualified according to gene similarity analysis (Jouzani et al., 2017). These genes are updated and listed in the website: [http://www.lifesci.sussex.ac.uk/home/Neil\\_Crickmore/Bt/intro.html](http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/intro.html). Polymerase Chain Reaction (PCR) is the most used method to characterize *B. thuringiensis* genes (Fané et al., 2017). Genetic diversity has focused on Enterobacterial Repetitive Intergenic Consensus (ERIC) elements and Repetitive Extragenic Palindromic (REP) sequences, also via PCR (Mishra et al., 2017). Repetitive Elements Polymorphism (REP-PCR) fingerprinting is commonly used to discriminate bacteria species analyzing the distribution of repetitive DNA sequences in several prokaryotic genome (Versalovic et al., 1991). This methodology uses specific primer sets for recognition of repetitive sequences that show inter-repetitive distances but also specific patterns among bacteria species and strains (Van Belkum et al., 1998), indicating that it represents a rapid shortcut for addressing the genetic relationship of unknown strains with the major known serovars (Cherif et al., 2007). Likewise, ERIC-PCR involves the use of primers composed of 22 nucleotides displaying high homology for repetitive intergenic sequences commonly present in all prokaryotic kingdoms (Versalovic et al., 1991).

*Spodoptera eridania* (Cramer) (Lepidoptera: Noctuidae) causes significant losses to soybean and cotton crops (Silvie et al., 2013). It is responsible for injuries to pods and leaves, leaf-loss, and reduction on yield and plant growth (Bernardi et al., 2014). Currently, the most widely used method for *Spodoptera* spp. control consists in the use of chemical pesticides such as: phosphate, carbamate, pyrethroid and growth regulator (CABI, 2018). *B. thuringiensis* may be an alternative to control this insect pest.

The main objective of this work was to characterize the toxicity towards *S. eridania* and the genetic diversity of 18 *B. thuringiensis* strains based on ERIC and REP sequences, and identifies possible grouping related strains.

## Material and methods

### *B. thuringiensis* strains

The study was carried out at the Laboratory of Biological Control, Maize and Sorghum, in Sete Lagoas, MG, Brazil. All strains have been previously tested against fall armyworm, *S. frugiperda* (Lepidoptera: Noctuidae J.E. Smith) (Valicente and Barreto, 2003; Valicente and Fonseca, 2010). A total of 18 *B. thuringiensis* strains were randomly selected from the *B. thuringiensis* collection and used in following experiments (Table 1).

The strains were grown in solid Luria-Bertani (LB) medium at 28°C + 2°C and the pH was adjusted to 7.5. After 72 hours, five colonies were chosen from each strain and inoculated into individual Petri dishes with solid LB medium. A loopful of each strain from two Petri dishes was inoculated into 1 mL of sterilized distilled water for DNA extraction, and the three remaining Petri dishes were used in bioassays.

### Insect feeding bioassays

The bioassay was carried out with neonate *S. eridania* larvae fed on artificial diet (Bowling, 1967), with 120 µL of *B. thuringiensis* suspension at the concentration of 10<sup>8</sup> spores/mL. Check treatment used artificial diet, and water experimental protocol was completely randomized block design consisting of 19 treatments with 4 replicates. Bioassay was composed with 4 caterpillars per replicate. Each neonate larvae were maintained individually in artificial diet plastic containers with acrylic lids. Mortality was evaluated after 7 days based on the average of

**Table 1**

Identification of *Bacillus thuringiensis* strains and its location sites in different regions of Brazil.

Identification of strain	Collection place
1089	Boa Esperança, MG**
939F	Teixeiras, MG
1043 N-V	Coqueiral, MG
1033B	Boa Esperança, MG
1058G	Guapé, MG
1039C2	Coqueiral, MG
1042B	Coqueiral, MG
970C	Teixeiras, MG
939FB	Teixeiras, MG
939FD	Teixeiras, MG
7B8	Limoeiro, AL***
986J	Sacramento, MG
976D	Uberaba, MG
813A	Viçosa, MG
T09	Paris, France*
1058A	Guapé, MG
1394	Caruaru, PE****
1093	Boa Esperança, MG

**Source:** Embrapa Maize and Sorghum Research Center/Biological Control Laboratory Data Collection. \* Kindly provided by Institute Pasteur; \*\* MG = Minas Gerais State; \*\*\* AL = Alagoas State; \*\*\*\* PE = Pernambuco State.

surviving caterpillars. Mortality rate was calculated using the number of dead caterpillars/ (number of surviving caterpillar x 100).

The toxicity results against neonate larvae were submitted to analysis of variance (ANOVA) in the Assistant software and the means compared by Scott-Knott test with 1% of significance ( $p < 0.01$ , F-48,27, F-crit 2.26).

### DNA extraction and PCR conditions

DNA extraction of the 18 *B. thuringiensis* isolates was performed according to the Wizard® Genomic DNA Purification Kit (Promega®, Madison, WI) procedure. DNA samples were quantified in ND-1000 UV/VIS spectrophotometer (NanoDrop Technologies, EUA), and diluted to a concentration of 50 ng/µL and stored at -2°C.

REP-PCR reactions used the following primers: REP1 5'-IIICGICGICATCIGGC-3', REP2 5'-ICGICTTATCIGGCCTAC-3', ERIC1 5'-ATGTAAGCTCCTGGGATTAC-3' e ERIC2 5'-AAGTAAGTACTGGGGTGAGCG-3' (Versalovic et al., 1994). The amplification reactions were carried out in a 25 µL reaction volume using 50 ng of genomic DNA, 1.5 mM MgCl<sub>2</sub>, 0.20 mM of dNTP, 0.5 µM of each primer and 2 U of Kapa Taq DNA polymerase (Sigma-Aldrich®, St. Louis, MO). The amplifications were performed in a thermocycler Mastercycler (Eppendorf, Hanburg, Germany) using the following program: 35 cycles at 94°C for 1 min, 48°C for 1 min for ERIC primers or 40°C for REP primers and 72°C for 6 min. A final extension step at 72°C for 10 min was added. The amplified fragments were separated by horizontal electrophoresis in a 1.5% agarose gel (TAE buffer and pH 8.0) at 80 V for 1 h. The gel was stained with Gel Red™ (Biotium, EUA), analyzed under UV light and photographed with a L-PIX system (Loccus biotecnologia, EUA).

Binary matrices were generated using the amplification products used as input data into Bionumerics software (Applied Maths, Belgium) and after the Pearson's correlation analysis. A similarity matrix was calculated from binary data using the Dice similarity coefficient. Clustering analysis was performed using this coefficient and the UPGMA (Unweighted Pair-Group Mean Average) with bootstrap of 1000 replicates to evaluate the consistency of the group. Finally, Bionumerics produced both the similarity matrix and dendrogram containing the 18 *B. thuringiensis* isolates.

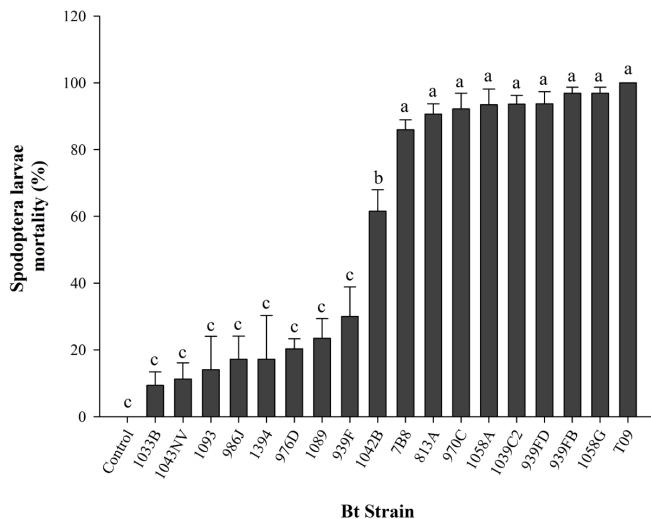
## Results

### Bioassays

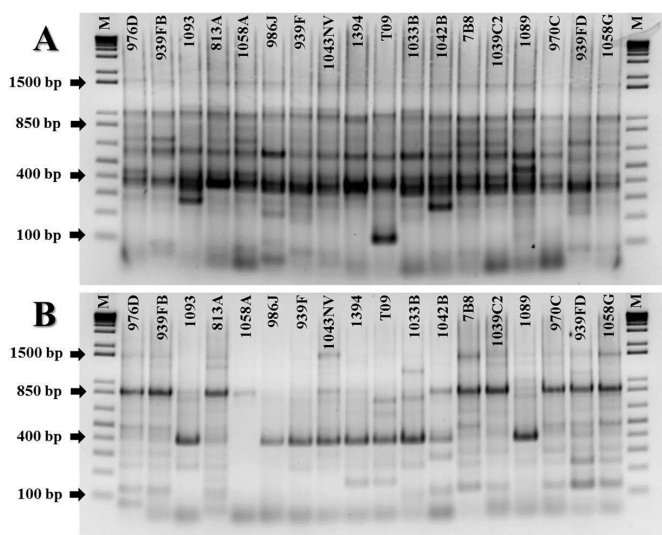
All *B. thuringiensis* strains led *S. eridania* larvae to death, but mortality level induced in caterpillars was isolate-dependent. The strains T09, 939FB, 1058G, 939FD, 1039C2, 1058A, 970C, 813A and 7B8 were responsible for the higher levels of mortality (superior to 85%). The 1042B strain induced an average mortality of approximately 60%, while the remaining strains 939F, 1089, 976D, 986J, 1394, 1093, 1043N-V and 1033B caused mortality ranged from 10 to 30%, without a statistical difference to the control (Figure 1).

### Molecular analysis

The pattern of REP-PCR and ERIC-PCR polymorphic bands was individually identified according to PCR product-specific migration profile after electrophoresis agarose gel. Electrophoresis profile of the PCR products amplified with ERIC primers (Figure 2A) exhibited the



**Figure 1** Mortality of *S. eridania* caterpillars caused by different *B. thuringiensis* (Bt) strains with a concentration of  $10^8$  spores/mL after 7 days of bioassay. Means followed by the same letter did not differ statistically by Scott-Knott test at 1% significance ( $p < 0.01$ ).



**Figure 2** Fingerprint patterns for ERIC-PCR (A) and REP-PCR (B) fragments of selected *B. thuringiensis* strains isolated in different locations. M = 1 kb Plus DNA ladder.

presence of 6 to 10 fragments which ranged from 100 bp to 1,500 bp, while the REP primers (Figure 2B) amplified 1 to 10 fragments per strain with sizes ranging between 50 and 2,000 bp.

A dendrogram was constructed through Pearson correlation for the genetic diversity analysis of ERIC and REP sequences with a 50% similarity cut-point. As a result, five groups were separated for this cut-point (Figure 3). The four strains clustered in group I induced mortality ranging from 9.37% (1033B) to 23.44% (1089). These strains were isolated from Boa Esperança (1033B, 1089 and 1093) and Sacramento (986J) samples, cities from the state of Minas Gerais. The mortality caused by strains clustered in group II ranged from 11.25% (1043NV) to 61.54% (1042B), and with exception of strain 1394, isolated in Pernambuco state, the others are from Coqueiral (1042B and 1043NV) and Teixeiras (939F), cities also located in Minas Gerais.

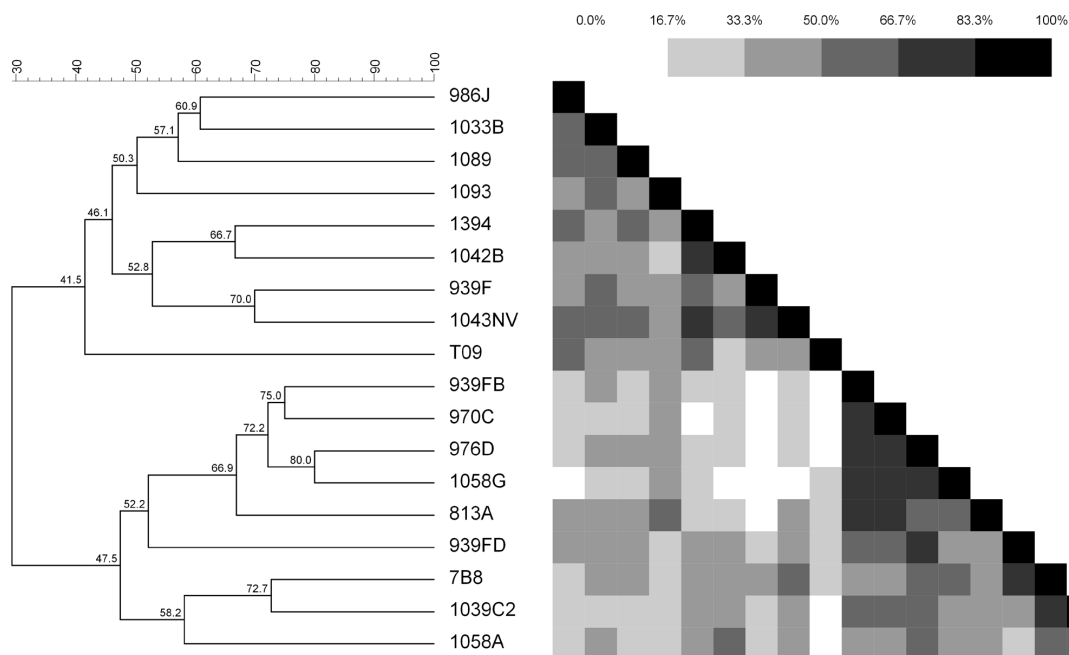
Group III is represented only for the T09 strain isolated in France, which caused 100% mortality on *S. eridania* larvae. The group IV was composed of six strains, and excluding the isolate 976D, obtained in Uberaba, Minas Gerais state, the other isolates (939FB, 970C, 1058G, 813A, and 939FD), also from cities in Minas Gerais, caused mortality rates superior to 90% upon larvae fed on artificial diets. The group V was clustered by the strains 7B8, 1039C2 and 1058A, isolated in the regions of Limoeiro (Alagoas state), and Coqueiral and Guapé (Minas Gerais), respectively. Similar to the strain T09 and the isolates in group IV, the strains at the group V were responsible for high mortality rates on larvae, with values vary from 85.94 to 93.65%.

## Discussion

According to our results, half of the *B. thuringiensis* strains tested were able to cause death to *S. eridania* larvae. It shows the high potential some specific *B. thuringiensis* strains have as biological control agents for pest insects like *Spodoptera* species, especially *S. eridania* in our case. Constanski et al. (2015) found that 3 strains exhibited toxicity higher than 90% against *S. eridania* and *S. frugiperda*. Similarly, dos Santos et al. (2009) identified among 100 *B. thuringiensis* strains some with a toxicity higher than 70% against *S. eridania*, *S. cosmioides* and *S. frugiperda* larvae. Valicente et al. (2010) also found many strains harboring different *cry1* genes that caused 100% mortality in *S. frugiperda* neonate larvae.

Monnerat et al. (2007) report the toxicity of a *B. thuringiensis* collection with 1,400 isolates against *S. frugiperda*, *Anticarsia gemmatalis* and *Plutella xylostella*. Twenty-seven *B. thuringiensis* isolates caused 100% mortality in *S. frugiperda*, *A. gemmatalis* and *P. xylostella* larvae. Faretto et al. (2007) also reported a high mortality rate in *S. frugiperda* larvae caused by 30% out of 115 *B. thuringiensis* strains. Huang et al. (2018) verified that the CAB109 strain caused mortality up to 55% in *S. exigua* larvae fed on diets containing *B. thuringiensis* suspension, and this strain also influenced the growth of *S. exigua* larvae in all instars. Valicente and Fonseca (2010) detected mortality around 95.8% caused by T09 strain against *Spodoptera* populations. Praça et al. (2004) found that in a group of 300 *B. thuringiensis* strains tested against 5 insect species, including *Spodoptera* sp., only 2 strains caused the death of all insects.

Researches based on the development or even identification of alternative methods for reducing the environmental impacts caused by conventional chemical compounds have increased in the scientific community (Glare and O'Callaghan, 2000). Considering the high toxicity induced by some *B. thuringiensis* isolates to different pest insects, make use of these bacteria as a biotechnological tool for pest control can be very beneficial to agriculture worldwide. Höfte and Whiteley (1989) explain that *B. thuringiensis* toxicity may be associated with the different shapes acquired by bacteria-produced parasporal inclusions. Moreover,



**Figure 3** Dendrogram and matrix similarity produced by software Bionumerics using agarose gel image as input data and a bootstrap of 1,000 replicates to estimate strains distribution. Data construction was supported by Pearson's correlation between ERIC and REP sequences, UPGMA cluster analysis and Dice similarity coefficient test.

variations observed in the susceptibility of *Spodoptera* populations to *B. thuringiensis*-produced toxins cannot be attributed only to the structural shape of endotoxins but also to genetic diversity found in *B. thuringiensis* communities (Hernández-Martínez et al., 2008).

Just a few papers have been published using REP-PCR to study the genetic diversity of *B. thuringiensis* isolates. Usually, REP and ERIC sequences are used for genetic diversity analysis of many organisms including bacteria (Ahmadi et al., 2018; Katara et al., 2012; Mishra et al., 2017), fish (Fernández-Álvarez et al., 2018), and plants (Rampadarath et al., 2015). REP and ERIC primers have been useful in distinguishing *B. thuringiensis* isolates found in different locations (Katara et al., 2012) and roots of various legumes (Mishra et al., 2017). Based on the similarity for individuals distributed in the same group, it was possible to see a small correlation between *B. thuringiensis* strain-induced mortality and collection place, even regarding some strains originally isolated at a particular region being clustered together after the genetic diversity analysis (Figure 3).

In an attempt to link the subspecies according to collection places and toxicity against *Spodoptera* larvae, Silva and Valicente (2013) analyzed 65 *B. thuringiensis* strains by using REP, ERIC and BOX, and it resulted in 55 fragments amplified in 10 population groups. *B. thuringiensis* isolates found in Goiás state showed a high similarity themselves when compared to isolates found in other Brazilian states, which showed a larger genetic distance. As a consequence, the correlation between the collection places and toxicity was influenced by this genetic divergence for the last group. Katara et al. (2012) report that despite REP-PCR and ERIC-PCR did not generate similar fragment patterns among 113 strains found at the same location in India, such techniques may still be useful for distinguishing *B. thuringiensis* strains. A high genetic diversity was also observed among *B. thuringiensis* isolates present in different regions of Mexico, in which 39 fingerprints were identified in 40 *B. thuringiensis* isolates using ERIC-PCR (García et al., 2015). Vilas-Bôas and Lemos (2004) observed a high genetic diversity of 218 *B. thuringiensis* isolates found in Brazil.

Our results suggest that the genetic diversity of *B. thuringiensis* may be suffering influence of both ecological factors and geographic distribution of strains which have likely gone through a process of adaptation to different habitats. Such genetic variability is a very important characteristic of *B. thuringiensis* strains as it enables bacteria to adapt to several environments (Galvis and Moreno, 2014). The insecticidal activity of the isolates indicates these strains could be considered as potential biological agent candidates for further bioassays, with future perspectives to apply the best-performance strains as bioproducts in areas threatened by *S. eridania* attacks.

Silva and Valicente (2013) also characterized the genetic diversity of 65 *B. thuringiensis* strains. Their results showed 1 to 4 fragments obtained for strains characterized by the REP primers. The fragment sizes ranged from 396 to 3,054 bp. By using ERIC primers, the number of fragments varied 1 to 9 with sizes ranging from 220 to 2,036 bp.

A recent study showed the molecular diversity of endophytic *B. thuringiensis* isolates from root nodules of legume plants using ERIC-PCR. Authors concluded that *B. thuringiensis* diversity may be related to different factors such as the host plant genotype, region weather, and soil conditions, including the soil microbial communities. Additionally, bacteria can be transported by previously contaminated sources such as air dust, rainfall, and *B. thuringiensis* toxin-killed insect's cadavers, which may be ingested by other living insects and animals capable of scattering *B. thuringiensis* through feces (Mishra et al., 2017).

Absence or presence of gene similarity could be associated not only to strain toxicity or collection places but also to other factors like the occurrence of *cry*, *cyt* and *vip* genes or even  $\beta$ -exotoxins. Future works will be carried out aiming to detect Cry, Cyt and Vip toxins on previously tested strains under in vitro conditions in order to check if the presence of these genes might be a factor correlated to strain genetic similarity. The identification of  $\beta$ -exotoxin, known for being widely toxic to many species, is also extremely relevant to proceed on works focused on this approach, as its absence would allow determining the feasibility of the putative strain as a new biological control agent followed by its insertion into the Integrated Pest Management (IPM).

REP and ERIC primers are useful for characterizing *B. thuringiensis* isolates. Furthermore, fingerprinting techniques used for bacterial population study are considered advantageous due to the simplicity, detection capacity of a wide range of sequences and production of consistent results. In our work, ERIC-PCR method was more informative than REP-PCR. Additionally, *B. thuringiensis* isolates collected in different habitats exhibited certain genetic diversity degree. Although there was just a small correlation between *B. thuringiensis*-collection place and toxicity level, some isolates were highly entomopathogenic for *S. eridania* larvae. This work will contribute to *S. eridania* biological control once some strains with high toxicity level could be employed in *B. thuringiensis*-based formulations, but also used as sources for prospecting further protein-expressing genes specifically toxic to lepidopterans.

### Conflicts of Interest

The authors declare no conflicts of interest. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

### Author contribution statement

This work was carried out in collaboration between all authors. Déborah Heloísa Bittencourt Machado, Kalynka Gabriella do Livramento, Wesley Pires Flausino Máximo and Bárbara França Negri designed the study, performed the statistical analysis, and wrote the first draft of the manuscript. Luciano Vilela Paiva and Fernando Hercos Valicente managed the literature searches. All authors read and approved the final version of the manuscript.

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