

Low Transmission Rates of Equine Infectious Anemia Virus (EIAV) in Foals Born To Seropositive Feral Mares Inhabiting the Amazon Delta Region Despite Climatic Conditions Supporting High Insect Vector Populations

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Abstract

Background

Marajó Island within in the Amazon River Delta supports numerous bands of feral equids including the genetically distinct Marajoara horses. Roughly 40% of the equids on the island are infected with the Equine infectious anemia virus (EIAV). In the absence of iatrogenic transmission, spread of this lentivirus is mediated mainly by hematophagous insects whose year-round prevalence on the island is supported by favorable climatic conditions. The euthanasia of all infected equids within the population is not a feasible strategy when the prevalence of the disease is high or in highly specialized or rare breeds of equid such as the Marajoara horse. Preservation of these animals is complicated by high rates of seropositivity with the potential for vertical transmission or insect mediated transmission following parturition of foal. Therefore, the aim of this study was to evaluate EIAV vertical and post-partum insect-mediated transmission rates among foals born to seropositive feral mares until natural weaning. Serum samples of foals born to seropositive feral mares from Soure municipality, within Marajó Island, were collected to investigate their serological status, using an indirect ELISAppg45 with positive samples being tested in the classical agar gel immunodiffusion (AGID) assay to confirm the results.

Results

Twenty-eight foals were sampled and their serological status was monitored over a 2-year period. Depending on the birth date, some of them were sampled up to six times. All foals remained with their respective mares until fully weaned, approximately 10 months of age, and just 2 of 28 foals (7.14%) in the study group became seropositive against EIAV.

Conclusion

The results showed that in most cases it is possible to obtain negative foals born to and eventually weaned by EIA positive mares, even in equatorial regions where substantial rainfall and high temperatures favor the proliferation of insect vectors.

Background

Marajó Island (Pará state) situated in the Amazon River delta is the world's largest fluvial land mass and home to approximately 30,000 equids [1]. Among this equine population are horses that are direct descendants of animals introduced into the region by Portuguese colonizers in the early Eighteenth Century [2]. After transportation from the temperate grasslands of Europe to the Amazon delta region these animals were confronted with a tropical savanna type environment where in addition to different plant nutrient sources they were subjected to harsh hot (30-32°C) and humid ($\geq 80\%$) conditions all year round. Moreover, the area has a high annual rainfall (2.800-3.400mm) with the majority falling between January and May resulting in the case of Marajó Island in more than two thirds of the landmass becoming inundated [3, 4]. Although many equids live as feral in the island, some of them have become essential for transportation and extensive livestock maintenance of cattle and buffalo herds, the most important economic activities in the region [2, 5].

The initial horse population was interbred with some other Portuguese breeds including Arabian and Alter Real and eventually became recognized as the distinctive Marajoara horse. One of the major characteristics of this breed is adaptation to adverse tropical conditions [2, 6]. Although originally geographically isolated by the island environment, these unique horses have been interbred with other horse breeds recently imported into the area, to improve the size, posture and appearance, once the most horse breeders have prioritized the phenotypic characteristics, without taking into account the adaptation to the hostile environment. Unfortunately this practice is becoming a major threat to the genetic integrity of the Marajoara horse breed [2, 7, 8]. Another highly specialized breed found on Marajó Island is the Puruca horse. This was generated by breeding Marajoara horses to Shetland ponies and selecting individuals with a maximum standard height of 1.18 meters and they suffer an even greater threat, because it constitutes a very small population and the crossbreeding has resulted in the progressive replacement of this one to a bigger animal, mischaracterizing the main pattern of the breed [2, 7, 8].

Therefore, the equid population of Marajó Island contains distinctive, rare horse breeds that should be preserved because they possess genetic characteristics uniquely associated with adaptation to the tropical environment of the Amazon River Delta. However, in addition to dilution of these genetic characteristics by interbreeding another major threat to equids inhabiting Marajó Island is Equine Infectious Anemia Virus (EIAV). In a survey conducted on the island of just 294 equids 46.26% were found to be seropositive for this virus [9] suggesting it is endemic in the region.

EIAV, that causes the equine infectious anemia (EIA), is classified within the *Lentivirus* genus of the *Orthoretrovirinae* subfamily, family *Retroviridae* (ICTV, 2021). The virus is equid specific being reported in horses, donkeys and mules where it produces a persistent infection. Although clinical signs following exposure to EIAV are highly variable ranging from a subclinical infection to death, in many cases the disease presents in the form of three distinct clinical phases. These consist of an “acute” stage characterized by pyrexia coupled with thrombocytopenia, a “chronic” phase consisting of recurrent disease episodes where pyrexia plus thrombocytopenia are associated with anemia, edema, depressed neurological reactions together with cachexia and finally an “inapparent” phase where overt disease signs are not observed. Approximately 90% of equids survive the acute and chronic phases and after a period of 8–12 months gradually transition to the asymptomatic state where although blood-associated viral loads are usually low they remain a potential source for viral transmission [11, 12].

EIAV is transmitted mainly via contaminated blood with iatrogenic mechanisms such the reuse of veterinary equipment especially hypodermic syringes being a major source of spread. However, while vertical transmission has been reported, the most important means of spread, in the absence of human intervention, is the feeding behavior of large hematophagous insects including horse flies (Tabanidae family, *Tabanus* or *Hybomitra* genus), deer flies (Tabanidae family, *Chrysops* genus) and perhaps less frequently, stable flies (Muscidae family, *Stomoxys* genus) [13]. Transmission by hematophagous flies does not involve EIAV replication in insect tissues and so the process is purely mechanical. It occurs when feeding on an infected individual is interrupted causing the fly to seek a second uninfected equid host on which to complete its blood meal. Therefore the risk of transmission is directly related to the number of infected animals, proximity between animals and density of vectors, the latter being determined by environmental factors, including high temperatures and humidity [14–16]. These climatic conditions typify the Amazon Delta region and therefore equid populations in the region are subjected to year round high insect vector populations that consist of many different tabanid species [14, 17].

In the absence of viable vaccines or other effective treatments control of EIAV is dependent on the identification and removal of infected subjects from the population. The latter is either accomplished by life-long quarantine or more

often by euthanasia. Although effective at reducing the overall incidence of EIAV in an equid population such methods, especially when involving euthanasia, are not compatible with rare breed preservation where the maintenance of genetic diversity is a priority. However, it has been found that foals born to and raised by EIAV seropositive mares can remain seronegative [18] suggesting the possibility that virally infected mares can be successfully incorporated into breeding programs. Obviously, if verified this observation will have significant implications for the preservation of rare or otherwise valuable equids in areas where EIAV is prevalent. However, the study of McConnico et al. (2000) was conducted in the United States where high insect vector populations are not continually present and the foals were separated early from their mothers [18]. Therefore, a longitudinal study was conducted to determine viral transmission rates in foals born to EIAV feral seropositive mares, under natural weaning conditions, inhabiting Marajó Island, a region with a humid tropical rainforest climate, year round high insect vector populations and different species composition compared to those found in North America.

Methods

Study area

All samples were collected from a single rural property comprising a mostly native rather than actively managed habitat of about 5,000 hectares in the municipality of Soure, located on the east coast of Marajó Island. This island is the largest (approximately 49,606 km²) member of the Marajó archipelago and is located in the Amazon River Delta at the northern boundary of Pará state (Brazil) [19]. Marajó Island is bordered on the west and northwest by the Amazon River mouth, the Atlantic Ocean to the northeast and by the Pará River, a tributary of the Amazon to the east (Fig. 1).

Samples

The study period was from January 2018 until January 2020 and involved 28 foals (26 horses [*Equus caballus*] and 2 mules [*Equus caballus* x *Equus asinus*]) born to mares that were or became seropositive for EIA, as shown in Table 1. Although the majority of the horse foals were of mixed breed, eight subjects were Marajoara horses (Table 1). During the course of this longitudinal study foals depending on birth date, were sampled up to six times to investigate their serological status. Most of the equids on the property (259/388) were not confined to pastures or subject to any form of conventional animal husbandry (including the equids here analyzed in this study), while a smaller portion (75/388) were used for livestock management by the farm workers, but none of them were submitted to conventional procedures, such as vaccination and dietary supplementation. In this way, they could be defined as living a “feral” existence in that they were free-ranging in the environment and with the exception of sample collection not subject to regular human intervention. Parturition occurred under natural conditions and foals remained with their mothers until weaned, which occurred at approximately 10 months of age. Blood was collected by jugular venipuncture in tubes without anticoagulant and transferred to 1.5mL microtubes. The material was stored refrigerated until tested in the laboratory. All animal experimentation was approved by the Ethics Committee of the Use of Animals of the Universidade Federal de Minas Gerais, under protocol number 171/2018.

Serologic assays

Antibodies to the EIAV gp45 transmembrane envelope glycoprotein were detected by indirect ELISA (ELISAppg45) as described by Naves et al. (2019) [20]. Briefly, serum samples were diluted at 1:100 and added (100µl/well) to 96-well ELISA plates (Nunc-Immuno Plate Maxisorp, USA) previously coated with a synthetic gp45 peptide (0.5µg/well). All serum samples were analyzed in duplicate. Following a 60 min incubation period at room temperature (RT), each well was washed three times in phosphate buffered saline containing 0.01% Tween 20 (PBS-Tween) prior to the

addition (100µl/well) of a rabbit anti-horse IgG peroxidase conjugate (Sigma-Aldrich, USA) diluted 1:5.000 in PBS-Tween plus nonfat dried milk 0.01%. The ELISA plates were incubated for 60 min at RT and washed 3 times before addition of (100µl/well) o-phenylenediamine (OPD) substrate (0.5mg/mL) containing 0.02% of 30 volume hydrogen-peroxide. Color development was permitted for 10 min at RT before the reaction was stopped using sulphuric acid (1M). Optical density was determined at 492 nm in a microplate reader (BioTek ELx800, USA).

All ELISAppg45 positive samples were re-tested using a commercially available agar gel immunodiffusion (AGID) test kit according to the manufacturer's recommendations (Bruch Laboratories, Brazil) to confirm the results. As recommended by the Brazilian regulatory authorities, only serum samples that reacted in both tests were classified as positive [21].

Results

During the course of this study blood samples for serological analysis were collected from 26 horse and 2 mule foals. However, the foals used in this study were of different estimated ages with half of the study group being born after first sample collection date in January 2018. Therefore foals were divided into three groups. Group A consisted of 14 foals born prior to January 2018, Group B comprised of 8 foals born before the July 2018 collection point while Group C contained 6 foals born prior to the July 2019 sample date (Table 1). At the time of the first sample collection date foals in Group A ranged in age from 1 to 11 months (Table 1) and so the 3 oldest (Foals 28, 46 and 165 in Table 1) were almost fully weaned and were negatives for EIAV. All the mares in this group except one (mare of Foal 24) were found to be seropositive to EIAV in a survey conducted in January 2018 (Table 1). Three foals in this group produced test positive reactions in both the ELISAppg45 and AGID assays. Foal 230 was found to possess antibodies to EIAV in the sample collected in January 2018 but gave test negative results at all time points from July 2018 until completion of the study in January 2020. In view of the fact this foal was estimated to be just 1 month old in January 2018 the test positive reactions produced by just the initial sample are almost certainly the result of maternal antibody transfer. However, Foals 45 and 158 that had estimated ages of 7 months in January 2018 and did not have detectable antibodies to EIAV at this first blood sample collection point were both seropositive in July 2018. In view of the ages of these foals and initial test negative results the presence of EIAV specific antibody in July 2018 strongly suggests exposure to the virus either just before or sometime after January 2018. Foal 24 stayed with his mare for a few more months and remained negative until the end of the experiment, even after the mare had tested positive in Jul/18.

Two Foals in Group B (398 and 422) produced test positive results at the March 2018 and July 2018 sample collection points respectively (Table 1). However, it is estimated that both foals were approximately 1 month of age at the time of collection and as they were seronegative at all subsequent sample dates it is most likely the initial positive reactions in ELISAppg45 and AGID were the result of maternal antibody transfer. A similar case of maternal antibody transfer can also be made for Mule Foal 454 in Group C in that although it tested positive when approximately 2 months of age (November 2018) it was seronegative by July 2019 and again in January 2020 (Table 1) Therefore collectively these results demonstrate that of 28 foals studied just 2 (7.14%) were exposed to EIAV.

Table 1
Age, breed, sample collection date and results of foals evaluated for EIA

Foal	Breed	Age (months) prior to initial sample date	Date seropositivity of mare confirmed	Month/year of sample collection (foals)					
				Jan/18	Mar/18	Jul/18	Nov/18	Jul/19	Jan/20
24	Crossbreed	1	Jul/18	Neg	ND	Neg	Neg	Neg	Neg
28	Crossbreed	10	Jan/18	Neg	ND	Neg	Neg	Neg	Neg
45	Crossbreed	7	Jan/18	Neg	Neg	Pos	ND	ND	ND
46	Crossbreed	11	Jan/18	Neg	ND	Neg	Neg	ND	ND
78	Marajoara	7	Jan/18	Neg	ND	Neg	Neg	Neg	Neg
89	Crossbreed	6	Jan/18	Neg	ND	Neg	Neg	Neg	Neg
137	Crossbreed	7	Jan/18	Neg	ND	Neg	Neg	Neg	Neg
154	Marajoara	8	Jan/18	Neg	ND	Neg	Neg	Neg	Neg
155	Crossbreed	7	Jan/18	Neg	ND	Neg	Neg	Neg	Neg
158 ¹	Marajoara	7	Jan/18	Neg	ND	Pos	ND	ND	ND
165	Crossbreed	10	Jan/18	Neg	ND	Neg	Neg	Neg	Neg
196	Crossbreed	6	Jan/18	Neg	ND	Neg	Neg	Neg	Neg
200	Crossbreed	6	Jan/18	Neg	ND	Neg	Neg	Neg	ND
230	Crossbreed	1	Jan/18	Pos	ND	Neg	Neg	Neg	Neg
398	Marajoara	1	Jan/18	–	Pos	Neg	Neg	Neg	Neg
404	Marajoara	1	Jan/18	–	Neg	Neg	Neg	Neg	Neg
420	Crossbreed	4	Jan/18	–	–	Neg	Neg	ND	ND
421	Crossbreed	4	Jul/18	–	–	Neg	Neg	Neg	Neg
422	Crossbreed	1	Jan/18	–	–	Pos	Neg	Neg	Neg
424	Crossbreed	3	Nov/18	–	–	Neg	Neg	Neg	Neg
436	Crossbreed	3	Jul/18	–	–	Neg	Neg	ND	ND
439	Crossbreed	2	Nov/18	–	–	Neg	Neg	Neg	Neg

Foot notes:

¹ Foal 158 presented a positive result in ELISAppg45 and negative in AGID in dez/2018 and in the following test it also became positive in AGID

ND: Not done

–: Foal had not borned at time of sample collection

Foal	Breed	Age (months) prior to initial sample date	Date seropositivity of mare confirmed	Month/year of sample collection (foals)					
				Jan/18	Mar/18	Jul/18	Nov/18	Jul/19	Jan/20
454	Mule	2	Jul/18	–	–	–	Pos	Neg	Neg
455	Marajoara	5	Jan/18	–	–	–	Neg	Neg	Neg
516	Marajoara	7	Jul/18	–	–	–	–	Neg	Neg
517	Marajoara	4	Jul/18	–	–	–	–	Neg	Neg
518	Crossbreed	3	Nov/18	–	–	–	–	Neg	Neg
519	Mule	4	Nov/18	–	–	–	–	Neg	Neg
Foot notes:									
¹ Foal 158 presented a positive result in ELISAppg45 and negative in AGID in dez/2018 and in the following test it also became positive in AGID									
ND: Not done									
–: Foal had not borned at time of sample collection									

Discussion

The Marajoara and Puruca horse breeds have adapted over centuries to survive in the adverse hot and humid conditions of the Marajó Archipelago. However while they were originally geographically isolated by their island environment they have been interbred with many other horse breeds recently introduced into the area. This has resulted in a reduction in the number of individuals possessing the complete compliment of unique genetic characteristics associated with these breeds and has put them at risk of extinction [2, 22]. However, efforts to preserve both the Marajoara and Puruca breeds are complicated by the fact EIAV is endemic on Marajó Island and that conventional methods of controlling this virus involving euthanasia of infected individuals are not feasible, especially when the incidence within the region's equid population probably exceeds 40% [9].

For the preservation of any endangered species or breed it is important to maintain as much genetic diversity as possible from the original population in order to avoid founder effects. These may manifest as undesirable genotypic or phenotypic traits and occur when a new population is established using too few individuals. Consequently, to ensure survival of the Marajoara and Puruca horses in their original form it may be desirable or even necessary to enroll EIAV seropositive mares in any restorative breeding program. However this would not be possible if there is a high risk of vertical transmission from mare to foal either *in utero*, during parturition or from the intake of colostrum and breast milk. In human populations infected with the related lentivirus, Human immunodeficiency virus (HIV) for example, it has been demonstrated the risk of vertical transmission from mother to neonate in the absence of antiretroviral drug therapy ranged from 15–35% [23]. Moreover, in addition to HIV it has been shown that other lentiviruses including the Small ruminant lentiviruses (SRLV) and Feline immunodeficiency virus (FIV) plus several other retroviruses such as Mouse mammary tumor virus (MMTV), Bovine leukemia virus (BLV) and Human T-cell leukemia virus type 1 (HTLV-1) are all efficiently transmitted via breast milk [24–28].

Although studies in horses are far from exhaustive it appears that EIAV can be transmitted *in utero* with an incidence of 100% (7/7) if the mares experience a clinical episode during gestation. However, the probability of vertical transmission decreases to less than 11% (5/45) if the mare remains asymptomatic. These foals developed clinical signs by 4 months of age, but were kept in the same place with another infected foal, because of this, the transmission may not have been from mare to foal, but horizontally through insect bites [29]. These observations suggest the high blood-associated viral loads that are invariably present during febrile episodes [15] significantly increase the probability that EIAV will cross the placenta. If, on the other hand, the infected mare retains its inapparent carrier status then the risk of transmission to the fetus either *in utero* or during parturition becomes diminished by almost at least 90%. In addition, it appears that while milk from EIAV positive mares contains virus that is infectious when inoculated intradermally into seronegative recipient horses the virus is not transmitted to foals during breastfeeding [30–32]. When taken together these early studies suggest the risk of vertical transmission in the case of EIAV inapparent carrier mares is significantly lower than in infections with related lentiviruses including HIV, FIV and SRLV. This prediction is supported by the results of this study and by previous work involving foals born to EIAV positive mares [16, 18, 33, 34]. Indeed while two foals (45 and 158) became seropositive during the course of this study this occurred when they were somewhere between 7 and 14 months of age. Therefore, vertical transmission is highly unlikely in these cases.

Although vertical transmission of EIAV to foals born to inapparent carrier mares appears to be a rare event they may still be exposed to the virus via the feeding behavior of hematophagous insects especially as they usually remain in close proximity to their mother's until fully weaned. To reduce the risk of insect mediated transmission Silva et al. (2001) weaned foals born to EIAV seropositive mares at 6 rather than 10 months of age and then segregated them from the rest of the herd by at least 200 meters [16]. Although these measures prevented exposure to EIAV, early weaning results in stress to both mares and their foals plus quarantine measures are resource and management intensive. However, an experiment conducted in the United States involving 12 feral foals, separated from their mothers at 3–4 months of age, demonstrated the latter remained seronegative even though being reared by their respective inapparent EIAV carrier mares in their first months of life. Furthermore insect vector populations were classified as high during the summer months of this project [18]. In this current study it is shown that 24 of 26 (92.86%) foals born to EIAV seropositive mares remained seronegative to this virus even staying a long period with their respective EIAV-seropositive mothers, in the case of all but 2 Group A subjects, over 2 years. This is despite living a feral existence, unlike the animals described by McConnico et al. (2000), in an environment where EIAV is endemic and the hot, humid tropical rainforest climate supports very high insect vector populations all year round [14, 18, 35].

The results reported here are in agreement with a survey conducted in the Pantanal Sul-matogrossense region of Brazil, where seropositivity rates in foals approximately 1 year of age were found to be only 3.3% despite 65.93% of the general equid population being serologically test positive for EIAV [34]. Although, in this survey the serological status of each foal's respective mare was not determined its findings coupled with results described above suggest foals have a relatively low risk of EIAV exposure. Interestingly, this is despite living in an environment that is predicted to be highly favorable for insect mediated transmission. A potential explanation for this might include protective effects from the transfer of EIAV-specific antibodies in colostrum. Initially these may be present at relatively high concentrations in serum of foals but decrease over time becoming undetectable by AGID at a mean age of 183 days [18]. However, to our knowledge the efficacy of colostrum antibodies at preventing EIAV infection has not been investigated. Therefore another protective factor might be the fact young foals have a more vigorous defensive response to the presence of hematophagous insects than adult equids. These defensive movements often cause the insect to relocate to a more passive host. It has for example been observed that in freely grazing horse

herds, foals have only 2.43% the tabanid burden found on older animals [36]. In addition, larger adult animals may be more visually appealing to hematophagous insects and are likely to produce higher concentrations of chemotactic substances, such as carbon dioxide [14].

Although antibody transfer in colostrum and the more aggressive defensive behavior to hematophagous insects may contribute towards the low incidence of EIAV infection reported in this and other studies [18, 34], blood associated viral burdens in inapparent carrier animals is also likely to be extremely important. Unfortunately, this has not been studied extensively especially in feral equid populations. In experimental situations clinical episodes of EIA are associated with viral loads that can exceed 10^6 horse infectious dose₅₀ (HID₅₀) per mL of peripheral blood. The mouthparts of a large hematophagous insect such as *Tabanus fuscicostatus* retain 10 ± 5 nL of blood after an interrupted blood meal that in turn should, in the case of feeding on febrile equids, equate to 5 to 15 infectious EIAV particles. This suggests that in theory spread of EIAV can occur following a single fly-bite on an equid with clinical signs of EIA although experimentally it has been shown that transmission by *T. fuscicostatus* under these circumstances occurred in 1 of 7 attempts. In contrast inapparent carriers have viremia titers of 1 HID₅₀ per ml of peripheral blood or less. Under these circumstances the numerical probability of transmission by *T. Fuscicostatus* following an interrupted blood meal is approximately 1 in 67,000-200,000 [37–39]. If these numbers apply to the situation in the field then it is predicted the risk of insect mediated transmission will be low even in situations with high insect vector populations unless feeding occurs on an equid experiencing clinical signs of EIA. Although further studies are required results detailed here suggest that inapparent EIAV carrier mares pose only a very low risk for viral transmission to their foals at all stages of life ranging from *in utero* development to becoming fully weaned.

Conclusion

In conclusion, evidence is presented that supports the argument that vertical transmission of EIAV is an infrequent event in foals born to asymptomatic EIAV seropositive mares. Moreover, insect mediated transmission rates from inapparent carrier mares to foals are low thereby enabling a foal to remain with its mother until fully weaned, even in conditions that are highly conducive for maintaining year-round high density insect vector populations. These findings demonstrate that it is possible to obtain EIAV-free foals even when born to seropositive mothers providing the mares do not have clinical signs of EIA. This information is important for preserving genetically valuable equid traits such as those found in the rare Marajoara and Puruca horse breeds that are currently threatened with extinction.

Abbreviations

AGID
agar gel immunodiffusion
BLV
Bovine leukemia virus
EIAV
Equine infectious anemia virus
EIA
equine infectious anemia
ELISA
enzyme linked immunosorbent assay
FIV

Feline immunodeficiency virus
HID₅₀
horse infectious dose₅₀
HIV
Human immunodeficiency virus
HTLV-1
Human T-cell leukemia virus type 1
MMTV
Mouse mammary tumor virus
PBS
phosphate buffered saline
RT
room temperature
SRLV
Small ruminant lentiviruses

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of the Use of Animals (protocol number 171/2018) of the Universidade Federal de Minas Gerais. All methods were conducted in accordance with relevant guidelines and regulations. Informed consent was obtained from the equids owner that was informed about the methods and purpose of the study.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare no conflict of interest.

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There were no funding resources for this study.

Authors' contributions

CFR designed the project, carried out the experiments and wrote the manuscript. RMV and RJFC contributed to laboratory testing, data analyses and revision of the manuscript. AMS, GPG, JGL and AGMS performed all sample collections and data acquisition. RFC contributed data analysis and substantively revised the manuscript. RCL and JKPR participated in the study conception, supervised the procedures and revised the study. All authors read and approved the final manuscript.

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Figures

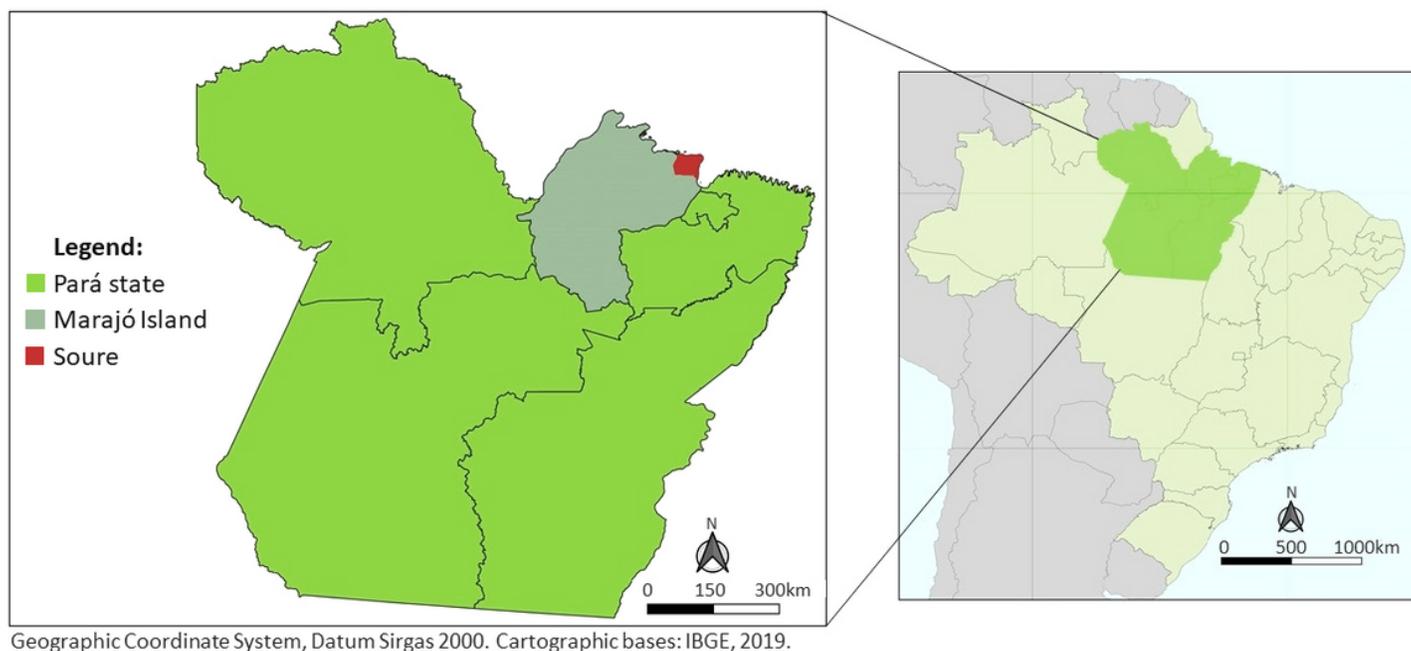


Figure 1

Geographic location of Soure municipality (red) within Marajó Island. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.