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Pathology, microbiology, and molecular evaluation of tissues from equids serologically positive for *Burkholderia mallei* in Midwestern Brazil[1]

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ABSTRACT.- Rocha L.S., Oliveira A.L.F., Arruda F.P., Pitchenin L.C., Dutra V., Nakazato L., Furlan F.H. & Colodel E.M. 2023. **Pathology, microbiology, and molecular evaluation of tissues from equids serologically positive for *Burkholderia mallei* in Midwestern Brazil.** *Pesquisa Veterinária Brasileira 43:e07172, 2023.* Faculdade de Medicina Veterinária, Universidade Federal de Mato Grosso, Av. Fernando Corrêa da Costa 2673, Bairro Boa Esperança, Cuiabá, MT 78068-900, Brazil. E-mail: edson.colodel@ufmt.br

Glanders is a disease caused by the bacterium *Burkholderia mallei* that primarily affects horses, mules and donkeys. The disease can cause lesions in the skin, lungs and several other organs. However, it often manifests as an asymptomatic disease. In Brazil, serological tests of high sensitivity and specificity are used to assist in the detection of antibodies against *B. mallei* and to contribute to the control of the disease. However, due to the mandatory euthanasia of seroreactive animals, equids with positive serology for *B. mallei* and asymptomatic generated great conflicts between breeders, veterinarians and diagnostic laboratories. This study clarifies the limitations of complementary diagnostic tests for detecting *B. mallei*. It describes the clinical, morphological and laboratory findings in 24 equines from different municipalities in the Mato Grosso State, Brazil, which reacted to the complement fixation test and were positive in the western blotting test for glanders. Data and tissue samples were collected from 24 horses for histological, microbiological and molecular analysis. In 23 horses, no clinical signs, morphological alterations, microbiological isolation, or molecular detection would characterize *B. mallei* infection. On the other hand, samples from an asymptomatic horse without lesional alterations showed sequence amplification compatible with *B. mallei* in the PCR. Considering that the infection by *B. mallei* is subject to the application of animal sanitary defense measures and that, by international requirement and national legislation, the serological results are tools that should support the sanitation procedures for the error of the bacteria in the Mato Grosso State, Brazil.

INDEX TERMS: *Burkholderia mallei*, glanders, complement fixation test, Equidae, Brazil.

RESUMO.- [**Avaliação patológica, microbiológica e molecular em amostras de tecidos de equídeos sorologicamente positivos para *Burkholderia mallei* no Centro-Oeste do Brasil.**] Mormo é uma enfermidade causada pela bactéria *Burkholderia mallei* que acomete primariamente cavalos, mulas e burros. A doença pode causar lesões na pele, pulmões e em diversos outros órgãos, entretanto frequentemente manifesta-se como uma enfermidade assintomática. No Brasil são utilizados testes sorológicos de elevada sensibilidade e especificidade para auxiliar na detecção de anticorpos contra *B. mallei* e contribuir para controle da doença. Porém, devido à obrigatoriedade da eutanásia de animais sororeagentes, os equídeos com sorologia positiva para *B. mallei* e assintomáticos geraram grandes embates entre criadores, médicos-veterinários e laboratórios de diagnóstico. Este trabalho esclarece as limitações dos testes diagnósticos complementares para detecção de *B. mallei* e descreve os achados clínicos, morfológicos e de exames laboratoriais em 24 equídeos, procedentes de diferentes

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municípios do estado de Mato Grosso, Brasil, que reagiram ao teste de fixação de complemento e foram positivos no teste de "western blotting" para mormo. Foram colhidos dados e amostras de tecidos de 24 equídeos para análise histológica, microbiológica e molecular. Em 23 equídeos não existiam sinais clínicos, alterações morfológicas, isolamento microbiológico ou detecção molecular que caracterizassem infecção por *B. mallei*. Por outro lado, amostras de um cavalo assintomático e sem alterações lesionais apresentaram amplificação de sequência compatível com *B. mallei* na PCR. Considerando que a infecção por *B. mallei* é passível da aplicação de medidas de defesa sanitária animal e que por exigência internacional e da legislação nacional, os resultados sorológicos são ferramentas que devem amparar os procedimentos de saneamento para erradicação da bactéria no estado de Mato Grosso, Brasil.

TERMOS DE INDEXAÇÃO: *Burkholderia mallei*, mormo, teste de fixação de complemento, equídeos, Brasil.

INTRODUCTION

Glanders is a bacterial zoonosis caused by *Burkholderia mallei* primarily affecting solipeds (Van Zandt et al. 2013). The disease is clinically characterized by nodular lesions on the skin (cutaneous form), mucous membranes or even in organs such as the lungs (Elschner et al. 2017). There is no vaccine available for glanders, and control requires testing suspected animals, screening clinically healthy equines, and eliminating those reactive to diagnostic tests (OIE 2018). An important differential diagnosis is melioidosis, a disease caused by the saprophytic microorganism *Burkholderia pseudomallei* (Limmathurotsakul et al. 2016).

Glanders is listed as a notifiable disease, and the diagnosis can be based on epidemiological, clinical, and lesional findings. It can be confirmed by serological, microbiological, and maleinization tests (Naureen et al. 2007) or molecular methods (Laroucau et al. 2021).

The "Ministério da Agricultura, Pecuária e Abastecimento" (Ministry of Agriculture, Livestock and Food Supply – MAPA) established the "Programa Nacional de Sanidade dos Equídeos" (National Equine Health Program – PNSE) to prevent, diagnose, control, and eradicate diseases that can harm equids (Brasil 2008).

The epidemiological and clinical situation of glanders in Mato Grosso State is little known, and due to the economic and health impact that the outbreaks of the disease have throughout Brazil, there is a need to evaluate the results of diagnostic tests in the face of regional particularities. We report the clinical, pathological, microbiological, and molecular findings in 24 cases of seroreactive horses in the complement fixation (CF) and western blotting (WB) tests for glanders performed from 2015 to 2017 in Mato Grosso State, Brazil.

MATERIALS AND METHODS

The results of CF and WB performed routinely by veterinarians registered in the PNSE or official veterinarians from the "Instituto de Defesa Agropecuária do Estado de e Mato Grosso" (Institute of Agricultural Defense of the State of Mato Grosso – INDEA, MT) were made available for this research.

Equidae serum was collected by veterinarians and sent to accredited or official laboratories that issued the CF results performed by the cold method and followed the protocols of the national legislation at the time (Brasil 2004a, 2004b, INDEA 2014).

On the property where there was at least one Equidae with a result other than negative (positive, anticomplementary, or inconclusive) CF, the serum of this animal was collected by official veterinarians from INDEA. Samples were also collected from all other equids older than six months on the property to perform CF and WB in the Official Laboratory (Brasil 2004b, 2018a). WB was only performed on sera with a result other than negative for CF. Equidae with positive WB results was humanely euthanized by INDEA veterinarians.

To make a figure with the distribution of the outbreaks, we used Geographic Coordinate System Datum SIRGAS 2000 and Cartographic Bases' IBGE (IBGE 2023).

Clinical and morphological evaluation. The preliminary assessment of the cases was carried out by the Laboratory of Veterinary Pathology of the "Universidade Federal do Mato Grosso" (LPV-UFMT).

Histological evaluation. Fragments of organs were collected during the necropsy of each case. All fragments were fixed in 10% formalin and processed routinely, embedded in paraffin, cut to 5µm, stained with hematoxylin and eosin and evaluated microscopically at the LPV-UFMT.

Microbiological analysis and techniques for the identification of *Burkholderia mallei*. Blood was collected from the jugular during the clinical examination, and fragments of nasal turbinates, lymph nodes, lung, liver, spleen and tracheal swabs collected during the necropsy were sent to the Veterinary Microbiology Laboratory (UFMT). Additionally, when cutaneous, pulmonary or other organs were present, their content was aseptically collected through aspiration puncture and submitted to microbiological examination. An aliquot of each sample was stored at negative 20°C for DNA extraction, and the remaining samples were cultured on 5% Sheep Blood Agar and Sabouraud Agar and incubated at 37°C for up to 72 hours. Colonies were subjected to Gram stain and routine biochemical tests for identification (Quinn et al. 1994, Silva et al. 2009). Compatible colonies were inoculated in 3mL of BHI Broth (Brain Heart Infusion) for growth at 37°C for 24 hours under agitation. The medium was centrifuged at 14,462G for 5 minutes, and the supernatant was discarded, leaving the precipitate that was used for DNA extraction.

DNA extraction and polymerase chain reaction (PCR). In 2mL microtubes, 1mL of lysis buffer (100mM NaCl, 25mM EDTA, 100mM Tris-HCl pH 8.0, 0.5% SDS, 0.1mg Proteinase K) was added, and the material to be extracted (0.025g for tissue fragments, swabs with the stems removed, 250µl of whole blood and precipitate from BHI broth) was kept at 56°C overnight. After lysis, the DNA was extracted according to the protocol described by Sambrook & Russell (2001) by the phenol and chloroform method. The DNA obtained was eluted in 30µl of Tris-HCl buffer and stored at -20°C until use. DNA quality and integrity were checked by 1,5% agarose gel electrophoresis. PCR consisted of 10ng of genomic DNA, 20µM of each oligonucleotide (Scholz et al. 2006), 2.5x 10x PCR buffer, 0.2mM dNTPs, 1U of Taq DNA Polymerase and ultrapure water q.s.p. final volume of 25µL. Amplification cycles were performed according to the adapted protocol of Scholz et al. (2006), generating a 989bp fragment. The mastermix in this study has been performed with the Eppendorf® brand, which is unavailable. Our solution was to adapt the reaction to achieve a similar limit of detection of two copies/reaction based on the dilution of genomic DNA (Scholz et al. 2006). In this case, our limit of detection was 2.3 copies/reaction. The PCR products were subjected to electrophoresis in a 1.0% agarose gel, stained with Gel Red™ (Biotium®) at 10V/cm and visualized in a ChemiDoc™ XRS photo documenter using Image Lab™ software. The molecular mass marker used was a 100bp ladder.

RESULTS

From 2015 to 2017, the LPV-UFMT team followed seven outbreaks in six municipalities in Mato Grosso State, Brazil (Fig.1). Twenty-one horses and three mules were confirmed to be serologically positive for *Burkholderia mallei* (Table 1).

The total number of positive results in CF ranged from zero (Focus 7) to 42.30% (Focus 6); however, the results from Focus 2 and 4 were unavailable. Although in Focus 7 there were no positives in CF, the focus had 46 sera with results other than negative (26 anticomplementary and 20 inconclusive results) and subsequently submitted to WB, which resulted in positives in three sera. The total number of positive WB results ranged from 06.52% (Focus 7) to 58.82% (Focus 5) (Table 1).

Nineteen cases in Foci 1, 2, 3, 5 and 7 were euthanized and included in the study. In Focus 4, there were five cases of glanders, but one of the horses died spontaneously of an undetermined cause before the scheduled date for going to the focus and euthanizing the positive animals in WB; therefore, as it was not necropsied, this horse was excluded from the study. In Focus 6, only one case of the 15 equines positive in WB, which died spontaneously, was necropsied and included in the study. The rest of the horses in Focus 6 were not followed up on because the owners had no authorization.

All 24 cases had a good body score, there was no history of clinical alterations such as those described in glanders outbreaks, and the average age of the equines was three and a half years old. During the evaluation, all cases were clinically healthy; however, Case 13 presented a discrete translucent serous nasal secretion that was collected; however, no microbiological growth or PCR amplification of *B. mallei* was obtained from this material.

On postmortem inspection, lymph node enlargement was noted in eight horses (Table 2). Case 20 reddened the palatine tonsils, and Case 9 had moderate and diffuse hyperemia of the nasal turbinates. Case 21 died due to colic syndrome associated with gastric dilatation and was necropsied 6 hours after death.

The period between equine blood collection for serological diagnosis through WB and necropsy ranged from 25 to 243 days (Table 2). No history of a previous disease or clinical evolution resembled glanders in horses or mules after the complementary serological diagnosis. In the evaluations close to euthanasia, no significant changes were noted during the clinical examination or even during the necropsy.

Fig.1. Distribution of municipalities where equids were serologically positive for *Burkholderia mallei* in Mato Grosso State, Brazil.

In the histological examination, no lesions compatible with glanders were observed. The equine lymph node volume increase was related to mild lymphoid cell hyperplasia and mild or mild neutrophilic infiltrate. No other important microscopic changes were noted in the other organs analyzed.

There was no isolation of *B. mallei* from any of the samples collected. In case 21, from samples of the palatine tonsil, it was possible to amplify a sequence compatible with *B. mallei* in the PCR (Table 2).

DISCUSSION

This study reported no clinical signs or morphological alterations such as those described in glanders in equids. CF and WB were initially used to enable the movement of equines to participate in events with agglomeration or change of ownership. Malik et al. (2015) suggest that the poor economic situation of owners is an important risk factor that causes animal husbandry and welfare to be neglected. However, the foci monitored in Mato Grosso State were related to equines raised for effective and economic purposes with the sale of horses, participation in equestrian events, and leisure or occasional work helping to manage cattle. In addition, the horses' history did not include intense daily work.

It is often reported that pyogranulomatous lesions in the lung, lymph nodes, skin, or liver are found even in asymptomatic animals (Kettle & Wenery 2016), emphasizing that postmortem examination is an important complementary diagnostic tool for glanders. At that moment, samples can be collected where alterations suggestive of *Burkholderia mallei* infection are noticed, which are more specific for microbiological isolation and molecular tests.

No microbiological culture of *B. mallei* was obtained in the cases of this study, as well as no reports or descriptions about the isolation of the bacterium in samples collected from equines in Mato Grosso State, Brazil. Bacterial isolation and identifying *B. mallei* from skin lesions and nasal exudates are considered the gold standard for diagnosing glanders (Elschner et al. 2019). There is difficulty in cultivating the bacteria from equines seropositive for glanders, as the chronicity of the lesions causes few colonies of *B. mallei* to be found in the samples collected, and contamination of the samples by environmental bacteria and fungi can still occur (Malik et al. 2015, Elschner et al. 2021). However, even in cases where the clinical and lesional alterations of glanders are not so evident, an alternative to increasing knowledge about the disease situation in the region is to intensify the referral of samples of cases with reactive serology to glanders for isolation or molecular detection of *B. mallei*. This procedure would be essential to support and confirm the diagnosis and avoid intense technical and legal controversies in cases of asymptomatic horses.

Identifying microorganisms through molecular techniques is widely used because it characterizes specific fragments of the infectious agent, not just the antibody. Although PCR is important for confirming glanders in equids with clinical signs, the test is not appropriate for prevalence studies, as the sensitivity in clinical samples is not yet known (OIE 2018). Suppose the sample is taken from a part of the animal without

B. mallei, the test will be negative (Elschner et al. 2017). For these reasons, a negative molecular result is not proof of the absence of *B. mallei* in the sample, and other diagnostic means must be applied for confirmation.

Several configurations have been designed for the molecular diagnosis of glanders, but there is still difficulty in choosing the appropriate gene to distinguish *B. mallei* and *Burkholderia pseudomallei* (Khan et al. 2013). A sequence known as IS407A disrupts the flagellar gene (fliP) that encodes flagellar P protein in *B. mallei*, rendering it immobile, and is present in all strains; however, it is not present in *B. pseudomallei* (Song et al. 2010). False-negative PCR results can occur in regions with new circulating strains of *B. mallei* or strains that have not been genetically identified. The importance of using PCR systems and serological systems for diagnosing glanders is highlighted when faced with situations such as this (Laroucau et al. 2021).

In samples collected from the palatine tonsil of an asymptomatic case of Focus 6, positive in CF and WB, who died due to colic syndrome associated with gastric dilatation, a compatible sequence of *B. mallei* was amplified in the PCR. This result can characterize the asymptomatic form that the disease can have, and the delay in euthanasia puts other animals and people at risk. It was not possible to evaluate the other horses in Focus 6, as those responsible took legal action, and there was an impediment to carrying out new tests to confirm the disease, in addition to the temporary suspension of euthanasia in cases of glanders in the focus. Despite the discussions about the zoonotic importance of the pathogen, with little epidemiological information, it is worth mentioning the existence of the infection in humans as reported in an 11-year-old boy, a horse caretaker, resident of the outskirts of Aracaju/SE (Brazil) who developed sepsis attributed to *B. mallei* (Santos Júnior et al. 2022). Given this situation, PCR has become an important method to complement the serological tests used in glanders control and eradication programs (Abreu et al. 2020).

Using serological tests for glanders diagnosis is the main challenge in Brazil (Abreu et al. 2020). Official tests include CF, WB, intrapalpebral maleinization (MI) and the enzyme immunosorbent assay (ELISA) (Brasil 2018a). The Mato Grosso State has the 6th largest troop in Brazil, with 283,480 horses

Table 1. Distribution of glanders serological tests performed in seven foci in municipalities in Mato Grosso State, Brazil

Focus	Municipality of focus in Mato Grosso State	Total sera sent to CF, and results obtained	% CF positive sera in the focus	Total sera sent to WB, and results obtained	% WB positive sera in focus
1	Jangada	37 equines and 2 mules Eq: anticomplementary CF: 1 serum Eq: inconclusive CF: 3 sera Eq: negative CF: 24 sera Eq: positive CF: 9 sera Mule: negative CF: 2 sera	23.07%	13 equines Eq: positive WB: 1 serum Eq: negative WB: 12 sera	7.69%
2	Acorizal	The number of reacting equines was not reported.	No information	6 horses and 1 mule Eq: negative WB: 4 sera Eq: positive WB: 2 sera Mule: inconclusive WB: 1 serum	28.57%
3	Rondonópolis	16 equines and 10 mules Eq: anticomplementary CF: 3 sera Eq: negative CF: 10 sera Eq: positive CF: 3 sera Mule: inconclusive CF: 2 Mule: negative CF: 6 sera Mule: positive CF: 2 sera	19.23%	6 equines and 4 mules Eq: inconclusive WB: 3 sera Eq: negative WB: 3 sera Mule: inconclusive WB: 1 sera Mule: positive WB: 3 sera	30%
4	Poconé	The number of reacting equines was not reported.	No information	15 equines Eq: negative WB: 10 sera Eq: positive WB: 5 sera*	33.33%
5	Poconé	74 equines Eq: negative CF: 57 sera Eq: positive CF: 17 sera	22.97%	17 equines Eq: inconclusive WB: 6 sera Eq: negative WB: 1 serum Eq: positive WB: 10 sera	58.82%
6	Cuiabá	49 equines and 3 mules Eq: anticomplementary CF: 3 sera Eq: negative CF: 22 sera Eq: positive CF: 20 sera Eq: inconclusive CF: 4 sera Mule: positive CF: 2 sera Mule: negative CF: 1 serum	42.30%	27 equines and 2 mules Eq: inconclusive WB: 12 sera Eq: negative WB: 2 sera Eq: positive WB: 13 sera Muar: Positive WB: 2 sera	44.82%
7	Nova Mutum	107 equines and 9 mules Eq: anticomplementary CF: 21 sera Eq: inconclusive CF: 16 sera Eq: negative CF: 66 sera Mule: anticomplementary CF: 5 sera Mule: inconclusive CF: 4 sera Mule: negative CF: 4 sera	00.00%	37 equines and 9 mules Eq: negative WB: 34 sera Eq: positive WB: 3 sera Mule: negative WB: 9 sera	6.52%

Eq = equine, CF = complement fixation, WB = western blotting; * One of these equines-positive WB died on the property and was not necropsied.

(IBGE 2017). Glanders was first reported in the Mato Grosso State in 2014 in the municipality of Vila Bela da Santíssima Trindade, with serological diagnosis based on CF (FAMATO 2014). From 2014 to 2019, Mato Grosso State recorded 115 cases, 18 in 2014, 69 in 2015, 11 in 2016, 16 in 2017, no in 2018, and one in 2019 (MAPA 2021).

Positive results in screening and complementary tests for glanders in asymptomatic equines generated disagreements between technicians, breeders, and diagnostic and inspection networks during the study. However, ELISA was included in the PNSE in 2018 to overcome the disadvantages of CF. From 2018 to 2020, both CF and ELISA can be used as screening tests, and since April 2020, ELISA has become the only screening test, and CF will be used for international transport (Brasil 2018a).

Different ELISA formats have been developed for the diagnosis of glanders. Indirect ELISA is a technique used in several countries. It has a sensitivity equivalent to or greater than CF, whereas ELISA with recombinant proteins has high sensitivity and specificity (Mota & Ribeiro 2015). Rocha et al. (2021) reported that a horse without clinical signs was diagnosed through indirect ELISA; later, the case started to show expectoration of mucopurulent secretion and edema of the hind limb, but even with suggestive signs of glanders and seropositivity in the indirect ELISA, the animal remained negative in the CF.

CF played an important role in the eradication of glanders in several countries, but there may be variation in sensitivity and specificity depending on the antigen and the methodology used to perform the test (Kettle & Wernery 2016); thus, inconsistent results may occur for confirmation of clinical cases and in prevalence studies and eradication programs (Fonseca-Rodríguez et al. 2019). On the other hand, WB is a specific technique capable of complementing CF (Elschner et al. 2011). However, further studies are still needed to clarify the importance and occurrence of other diseases that may cause cross-reactions in the tests used to diagnose glanders in Mato Grosso State, among them melioidosis. In regions

Table 2. Results of complementary exams of equines with positive serology for glanders in Mato Grosso State, Brazil

Case	Focus	Species	Gender	Age (years)	Days interval between blood collection for WB and necropsy	Most important clinical and lesional findings	CF	WB	*Burkholderia mallei* isolation	*B. mallei* PCR
1	1	Equine	M	3	25	No alteration	P	P	N	N
2	2	Equine	M	2	28	No alteration	Un.	P	N	N
3	2	Equine	M	11	28	No alteration	Un.	P	N	N
4	3	Mule	M	14	62	No alteration	P	P	N	N
5	3	Mule	M	12	62	No alteration	In.	P	N	N
6	3	Mule	M	12	62	No alteration	P	P	N	N
7	4	Equine	M	4	243	Submandibular, retropharyngeal, and mesenteric lymph nodes slightly increased.	Un.	P	N	N
8	4	Equine	M	6	243	Prescapular and submandibular lymph nodes slightly increased.	Un.	P	N	N
9	4	Equine	M	6	243	Submandibular lymph nodes are slightly enlarged. Moderate and diffuse hyperemia of the nasal turbinates.	Un.	P	N	N
10	4	Equine	M	6	243	Submandibular lymph nodes are slightly enlarged.	Un.	P	N	N
11	5	Equine	M	2	136	Submandibular lymph nodes are slightly enlarged.	P	P	N	N
12	5	Equine	M	3	136	Submandibular lymph nodes are slightly enlarged.	P	P	N	N
13	5	Equine	F	8	136	Slightly enlarged prescapular and mesenteric lymph nodes. Discrete translucent nasal secretion.	P	P	N	N
14	5	Equine	F	3	136	No alteration	P	P	N	N
15	5	Equine	F	6	136	No alteration	P	P	N	N
16	5	Equine	F	2	136	No alteration	P	P	N	N
17	5	Equine	M	1	136	No alteration	P	P	N	N
18	5	Equine	F	3	136	No alteration	P	P	N	N
19	5	Equine	F	2	136	Enlarged mediastinal lymph nodes.	P	P	N	N
20	5	Equine	M	2	136	Moderate reddening of the palatine tonsils.	P	P	N	N
21	6	Equine	M	5	211	Gastric dilatation with compacted contents; nasal turbinates diffuse and intensely red.	P	P	N	**P**
22	7	Equine	M	3	99	No alteration	P	P	N	N
23	7	Equine	F	3	99	No alteration	P	P	N	N
24	7	Equine	F	2	99	No alteration	P	P	N	N

F = female, M = male, P = positive result, N = negative result, CF = complement fixation, WB = western blotting, PCR = polymerase chain reaction, Un. = uninformed, In. = inconclusive.

where melioidosis is endemic, these serological tests cannot differentiate between *B. pseudomallei* and *B. mallei* infection due to the close phylogenetic relationship between the bacteria (Elschner et al. 2019).

Glanders is a notifiable disease, and for the sanitation of the outbreaks, euthanasia of seropositive animals must be performed (Brasil 2018b). This can generate controversy with horse breeders, especially when it involves asymptomatic horses, so it is necessary to promote serological studies on the disease, expand the investigation into reasons for seroconversion in horses with positive serology for glanders, and encourage clinical and lesional evaluation by associating histological, microbiological and molecular tests to minimize economic losses in the equine production chain.

CONCLUSIONS

In 23 equines, there was no glanders characterization based on clinical and lesional inspection or microbiological, histological and molecular techniques. In one asymptomatic horse without morphological changes, there was PCR amplification of a sequence compatible with *Burkholderia mallei*.

These findings show that the *B. mallei* positive serological reaction in the absence of characteristic clinical or lesional disease makes the subject sensitive to be addressed.

Therefore, the use of techniques for diagnostic complementation must be intensified to strengthen the basis of action of the activities of the National Equine Health Program.

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