

BURKHOLDERIA MALLEI ISOLATION FROM MILK OF A MARE AND EVIDENCE OF CONGENITAL TRANSMISSION OF GLANDERS IN EQUIDS: CASE REPORTS

ISOLAMENTO DE BURKHOLDERIA MALLEI DE LEITE DE ÉGUA E EVIDÊNCIA DE TRANSMISSÃO CONGÊNITA DE MORMO EM EQUINOS: RELATOS DE CASOS

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SUMMARY

This is the first report of *Burkholderia mallei* isolation in milk from a mare diagnosed with glanders and of transplacental transmission of the bacteria in a seropositive pregnant mare, both from São Paulo State, Brazil, 2016 and 2019. After necropsy, the horses were evaluated for macroscopic lesions, and organ samples were removed for histological, bacteriological, and molecular analysis. *B.mallei* was identified in milk samples by culture isolation and PCR, whereas in the fetus of the pregnant mare by PCR of the stomach fluid. These findings will contribute to the epidemiological knowledge of glanders life cycle in Brazil.

KEY-WORDS: Brazil. Notifiable disease. Farcy. Solipeds. Histopathology. Flagelin P.

RESUMO

Este é o primeiro relato de isolamento de *Burkholderia mallei* no leite de uma égua com diagnóstico de mormo e de transmissão transplacentária da bactéria em uma égua prenhe soropositiva, ambas do Estado de São Paulo, Brasil, 2016 e 2019. Após a necropsia, os cavalos foi verificada a presença de lesões macroscópicas e amostras de órgãos foram coletadas para posterior análise histológica, bacteriológica e molecular. *B.mallei* foi identificada em amostras de leite por isolamento bacteriano e PCR, enquanto no feto de égua prenhe por PCR do fluido estomacal. Esses achados contribuirão para o conhecimento epidemiológico do ciclo de vida do mormo no Brasil.

PALAVRAS-CHAVE: Brasil. Doença de notificação. Farcy. Solípedes. Histopatologia. Flagelina P

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INTRODUCTION

Glanders is a disease caused by *Burkholderia mallei*, a Gram-negative facultative intracellular bacillus (ACHA; SZYFRES, 2003). It is a potentially fatal infectious disease in solipeds (horses, mules, and donkeys) and may cause severe clinical signs in other animals including camels, bears, wolves, and dogs (OIE, 2018). The pathogen is able also to infect humans. Infection occurs primarily via the digestive tract, but can occur through respiratory, genital, and cutaneous routes, and the disease may be acute, subacute, or chronic. The disease usually becomes chronic in horses, and they can live for years with the infection without exhibiting clinical manifestation (ACHA; SZYFRES, 2003; MOTA et al., 2000; OIE, 2018). Previous studies (KHAN et al., 2013) established the transmission of the agent through direct contact of a naive animal with nasal discharge or material from skin pustules and ulcers of infected horses, including dried particles eliminated as aerosols. Indirectly, the pathogen may be transmitted through fomites contaminated with the bacteria, such as on veterinary equipment, water troughs, feed mangers, grooming tools, or hoof trimmings, even experimentally induced through mechanical vectors (housefly).

Clinical signs in horses include febrile episodes, debility, nasal discharge, coughing, and dyspnea, as well as nodular lesions that evolve into ulcers and heal to form star-shaped lesions (OIE, 2020). Glanders is currently endemic throughout Brazil and little progress has been made in epidemiology, molecular biology, or control of this important disease (FALCÃO et al., 2019). Mota et al. (2000) reported cases of glanders in Pernambuco and Alagoas States.

Glanders is a notifiable disease to the World Organization for Animal Health (OIE, 2018). In Brazil, it is included in the National Equine Health Program (PNSE) coordinated by the Ministry of Agriculture, Livestock, and Supply (Ministério da Agricultura, Pecuária e Abastecimento – MAPA, BRAZIL), the objective of which is to create strategies for epidemiological and zoosanitary surveillance and prophylaxis and eradication of diseases affecting equines. In cases of seropositivity, horses must be euthanized (BRAZIL, 2018). The goal of the present study was to describe aspects of glanders in two horses that were euthanized after confirmation of positive diagnosis in a screening test by the cold complement fixation test (CFT) and confirmed by western blot (WB) performed in a government laboratory, in accordance with Brazilian legislation (BRAZIL, 2004, 2018).

Due to the importance of expanding the knowledge of horizontal and vertical transmission of *B. mallei*, we investigated its presence in milk of a lactating mare with glanders and in tissue samples and stomach fluid from a fetus of nine months' gestation.

CASE REPORT

The study was conducted in compliance with criteria of the Ethics Committee on Animal Experiments of the Biological Institute, protocol registration number

CETEA-IB 157/17. Samples were obtained from two horses identified with clinical symptoms in outbreaks of glanders in São Paulo State Brazil in 2016 and 2019.

Horse #1, a six-year-old mare at approximately nine months' gestation, positive in CFT and WB, exhibited cervical lymph node enlargement and small cutaneous cervical abscesses. During necropsy, tissue samples were collected from lungs, mediastinal and mesenteric lymph nodes, heart, spleen, kidney, liver, and the central nervous system. The uterus and placenta were removed and the fetus necropsied to collect umbilical cord, lungs, heart, kidney, spleen, liver, and stomach fluid. The fetus presented icteric ocular mucosa and yellowish miliary lesions in the umbilical cord.

Horse #2 was an 8-year-old lactating mare, four months post-foaling, positive in CFT and WB, that showed bilateral ocular ulcers (Figure 1A), bilateral catarrhal ocular discharge, and intense bilateral catarrhal nasal discharge. At necropsy, samples of trachea, mediastinal lymph nodes, lungs, liver, spleen, kidney, ocular globe, and milk were collected.

Tissue samples from both horses and the fetus were separated in duplicate, one stored in 10% buffered formalin (v/v) for pathology analysis and the second placed in a plastic bag and held at 2–8°C for bacteriological testing, attending Brazilian legislation (BRAZIL, 2018) and PCR. Samples were transported to the General Bacteriology Laboratory at the Biological Institute, São Paulo, Brazil, in an isothermal box, category B (UN3373) for the transport of biological substances (WHO, 2004).

For histological analysis, after 48h fixation in 10% buffered formalin, the organ samples were processed for histology using standard protocols and 3 µm sections stained with hematoxylin and eosin were examined with 100x oil immersion microscopy.

The bagged organ and milk samples were suspended 1:5 w/vin sterile 0.85% saline solution, and 10 µL of the suspension was seeded onto 5% sheep blood agar with 5% glycerin and 2500 IU potassium benzyl penicillin (selective medium for *B. mallei*) and incubated for 48–72 h at 37°C (MERWYN et al., 2010; QUINN et al., 2011; WINN et al., 2008). Morphological characteristics of the resultant bacterial colonies were recorded, including size, shape, color, and presence and type of hemolysis. The colonies were examined after Gram negative staining under oil immersion for morphology, cell distribution patterns, and staining characteristics. *B. mallei* was identified according to previously described (QUINN et al., 2011; WINN et al., 2008) biochemical protocols assessing catalase, oxidase, indole, nitrate reduction, results of the Voges-Proskauer test, motility, and fermentation of sugars.

After growth the colonies showing phenotype suggestive of *Burkholderia* spp., such as Gram-negative bacilli staining and characterization by fermentation of sugars as non-fermentative, were resuspended in 0.85% saline, inactivated at 100°C for 10 min and stored at -20°C for molecular analysis. DNA from these colonies and clinical samples of the necropsied animals (saline suspension of organs and fluids), previously separated

during the necropsy, were extracted using the Quick DNA Miniprep kit Zymo Research, according to the manufacturer's instructions. PCR was performed for detection of the flagellin *P. B. mallei* gene (*fliP*) using primers modified from Scholz et al. (2006), Bma-IS407-flip-F (5'TCAGGTTTGTATGCTCGG3'), and Tomaso et al. (2006), Bma-flip-R (5'GCCCCGACGAGCACCTGATT 3'), yielding a 528 bp fragment. Amplification of both clinical samples and bacterial colonies for *B. mallei* detection was performed using 10 µL of sample and 40 µL of a mixture of 1.25 U Taq DNA polymerase, 200 µM of each dNTP, buffer (10 mMTris-HCl, pH 8.0, 50 mMKCl), 2 mM MgCl₂, and 5 pmol/µL of each primer subjected to the following conditions: initial denaturation of 95°C for 4 min followed by 30 cycles of denaturation at 94°C for 1 min, hybridization at 60°C for 1 min, and extension at 72°C for 1 min, with a final extension of 72°C for 7 min. A strain of *B. mallei* INCQS 00115 (ATCC 15310) from the Collection of Reference Microorganisms in Sanitary Surveillance, FIOCRUZ-INCQS, Rio de Janeiro was used as positive control, and sterile deionized water as a negative control.

The amplified products were submitted to 1% agarose gel electrophoresis with added 10,000X red gel (Uniscence) at 1:125. Visualization of the bands was

performed with an ultraviolet light transducer (Ultra-Lum EA-40 UV).

RESULTS AND DISCUSSION

Necropsy Findings

Horse #1: fibrous visceral pleura with petechiae and suffusions, purulent lymphadenitis of the mediastinal lymph nodes, fibrin deposition in the peritoneal diaphragm, lemon-yellow liquid in the abdominal cavity, discrete petechiae and fibrin deposits in the splenic capsule, and a single miliary lesion in the kidney capsule.

The fetus of horse #1: multiple military lesions and enlarged veins in the placenta (Figure 1B), subcutaneous edema and jaundiced subcutaneous fat, pleural petechiae and pulmonary congestion, lemon-yellow pericardial fluid, petechiae in the visceral epicardium in the left ventricle, lemon-yellow peritoneal fluid, splenomegaly, and white pulp reaction in the spleen.

Horse #2: intense catarrhal discharge in trachea, several military and hemorrhagic lesions, fibrous thickened pleura (Figure 2) and necrotic areas in the lungs, enlargement of mediastinal lymph nodes, small abscesses in liver and kidney, and splenomegaly.



Figure 1 - (A) Horse #2(8-year-old lactating mare) seropositive for glanders showing ocular ulcer; (B) placenta from the fetus of horse #1 with enlarged veins (white arrows) and multiple military lesions (yellow arrows).

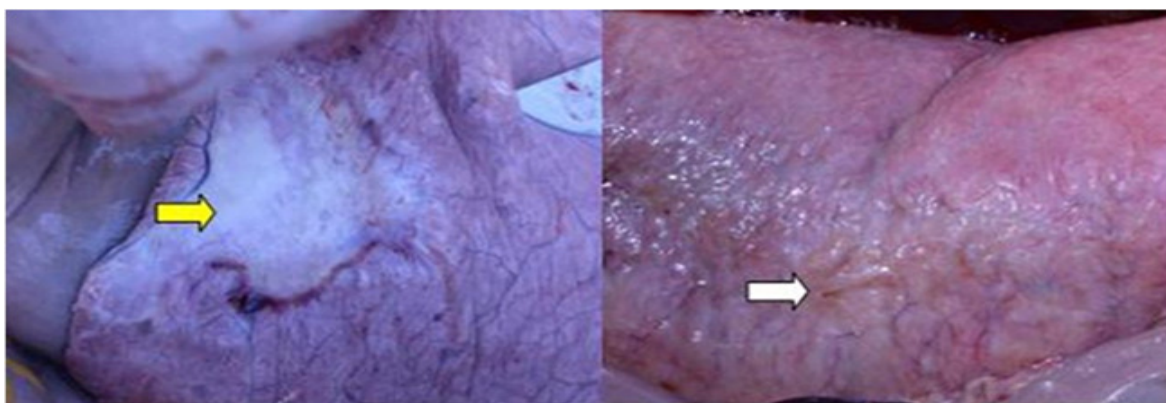


Figure 02 - Gross pathology findings in horses with glanders. Miliary and hemorrhagic lesions in lung from horse #2 (yellow arrow); fibrous thickened pleura in horse #1 (white arrow).

Microscopic findings

Horse #1: purulent pneumonia with multiple abscesses, pulmonary granulomas with Langhans giant cells (Figure 3), hemorrhagic foci in the liver parenchyma, hemorrhagic foci in the splenic capsule, discrete reaction of splenic white pulp, thymus and lymph nodes with intense lymphoid reaction and

hemorrhagic foci, purulent multifocal hepatitis, moderate and non-purulent membrane-proliferative glomerulonephritis, moderate splenic hemosiderosis. Diaphragm with peritoneal thickening due to fibrosis, dilated lymphatic vessels, intense mixed inflammatory infiltrate.

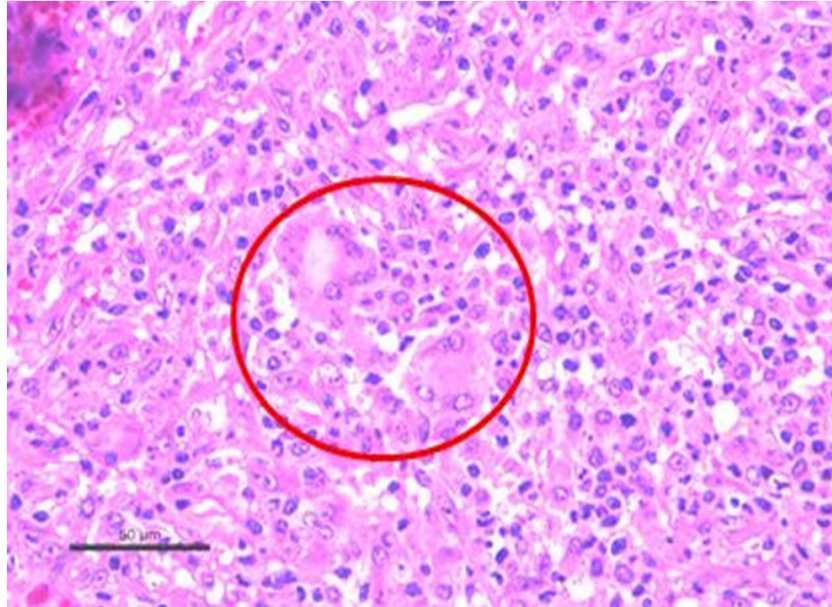


Figure 03 - Lesions found in the lung of horse with glanders by histology (circle): interstitial giant Langhans cells (HE, 400x)

Fetus from horse #1: pleural edema, multifocal hemorrhage (liver, lymph nodes, placenta, adrenal cortex), hyper-reactive lymphoid organs, intense reaction of splenic white pulp, and purulent inflammatory foci in the placenta and umbilical cord.

Horse #2: Purulent granulomatous conjunctivitis, purulent granulomatous pleuropneumonia with giant Langhans cells, purulent tracheitis, mediastinal lymph nodes with caseous lymphadenitis, purulent hepatic granulomas, non-purulent pseudomembranous glomerulonephritis, moderate splenic white pulp reaction, large splenic granuloma surrounded by epithelioid cells and fibrous capsule.

To our knowledge, there is no previous description of macroscopic and microscopic lesions in an equine fetus infected with *B.mallei* for comparison purposes, but the lesions presenting in this study are indicative of an infectious disease characterized by pyogranulomatous inflammation (MCGAVIN; ZACHARY, 2012). Based on location of lesions, glanders is classified as nasal, pulmonary, or cutaneous (OIE, 2020).

The observations reported in horses #1 and #2 are consistent with pulmonary glanders. Pyogranulomatous ulcerative lesions have been previously reported in the submucosa of the pharynx, larynx, and tracheal glanders (CASWELL; WILLIAMS, 2016). These lesions ulcerate, releasing copious quantities of *B. mallei*-containing exudate into the nasal cavity and reaching the lower respiratory tract. The lungs may also contain numerous miliary nodules, randomly distributed

in one or more pulmonary lobes. Microscopically, the nodules appear as typical chronic granulomas composed of a necrotic center surrounded by a thick band of connective tissue infiltrated with numerous macrophages, some giant cells, lymphocytes, and plasma cells. Pyogranulomatous nodules may occasionally be observed in kidney, spleen (WITTIG et al., 2006), and liver (OIE, 2020) as we have reported here. The pyogranulomatous conjunctivitis in horse #2 was a novel observation of the disease. Guinea pigs and rabbits experimentally infected with *B. mallei* exhibited proliferation of eosinophils, lymphocytes, and polymorphonucleated cells in the cornea (DUVAL; WHITE, 1907).

Bacterial cultures

A total of 23 samples were cultured for bacteria isolation: nine from horse #1, six from the fetus of horse #1, and eight from horse #2 (Table 1). Only the milk sample from horse #2 showed growth of *B. mallei* in a blood enriched medium (selective), suggesting that using this protocol could be more appropriate to isolation of *B. mallei* compared with other conventional isolation methods (MERWYN et al., 2010). Growth of *Bacillus* spp., *Staphylococcus* spp., *Escherichia coli*, *Pantoea agglomerans*, and yeasts (contaminants) was observed, with eight samples showing no growth after bacteriological culture. Due to the varying numbers of bacteria present in tissues depending on acute, chronic, or subclinical stage, *B. mallei* isolation may be challenging (SCHOLZ et al., 2006).

PCR

Of the 23 samples from the two necropsied horses and the fetus tested by PCR, eleven (48%) showed

B. mallei positive results (Table 1), including the milk sample, in which *B. mallei* growth in culture was also observed.

Table 1 - Results of microbial culture and PCR analysis of tissue samples using biochemical protocol and primers to detect the flagellin (*fliP*) gene of *B. mallei*.

Horse	Organ sample	Bacteriological culture	PCR
#1	Lung	<i>Staphylococcus</i> sp.	positive
	Lymph node	No growth	negative
	Heart	No growth	positive
	Liver	<i>Staphylococcus</i> sp., <i>Bacillus</i> sp.	positive
	Kidney	<i>Staphylococcus</i> sp.	positive
	Central Nervous System	<i>Bacillus</i> sp.	negative
	Placenta	<i>Staphylococcus</i> sp., <i>Bacillus</i> sp., <i>Pantoea agglomerans</i>	positive
	Umbilical cord	<i>Staphylococcus</i> spp.	positive
	Spleen	<i>Bacillus</i> spp.	positive
	Fetus from #1	Lung	<i>Staphylococcus</i> sp., <i>Bacillus</i> sp.
Spleen		<i>Staphylococcus</i> sp., <i>Bacillus</i> sp.	negative
Heart		<i>Staphylococcus</i> sp., <i>Bacillus</i> sp., <i>Escherichia coli</i>	positive
Stomach contents		No growth	positive
Liver		<i>Staphylococcus</i> sp., <i>Bacillus</i> sp.	positive
Kidney		<i>Staphylococcus</i> sp., <i>Bacillus</i> sp.	negative
#2		Mediastinal lymph node	Yeast
	Lung	No growth	negative
	Kidney	No growth	negative
	Liver	No growth	negative
	Heart	No growth	negative
	Spleen	No growth	negative
	Ocular globe	<i>Staphylococcus</i> spp., <i>Enterobacter cloacae</i>	negative
	Milk	<i>Burkholderia mallei</i> , <i>Bacillus</i> sp.	positive

The results of molecular analysis showed evidence of congenital transmission (PCR positive for fetal stomach fluid, heart, and liver), suggesting importance of clinical and serological monitoring of foals born to mares diagnosed positive after giving birth; since in Brazil, pregnant mares positive for glanders are euthanized along with the fetus and followed to at least six months of age. According to current PNSE guidelines, equines younger than six months are not included in the serological diagnosis for glanders, due to possible presence of colostral antibodies. Equine placenta is classified as epitheliochorial, and does not permit the passage of maternal immunoglobulins to the fetus. However, equine fetuses demonstrate partial immunocompetence, which means that antibodies can be detected in their sera following a congenital bacterial infection (SHEORAN et al., 2000). To improve the control of glanders in a herd, congenital transmission should be considered, with analysis of foal blood samples collected before the ingestion of colostrum.

CONCLUSION

The detection of *B. mallei* in milk of a glanders-positive mare (bacterial isolation and PCR) suggests a lactogenic transmission route of the bacteria in glanders infection and highlights the importance of

considering foals in the prevention of disease. It remains to be determined how long colostral immunity plays a part in the protection of foals and the point at which young horses become susceptible to bacteria by ingestion of milk. This route of the transmission needs to be further investigated to better describe the epidemiology of glanders, especially the *B. mallei* field strain viability in congenital transmission and infection via milk. In addition, to our knowledge, we describe for the first time macroscopic and microscopic lesions in an equine fetus infected with *B. mallei*.

CONFLICTS OF INTEREST

None to declare.

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