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Systematic monitoring of glanders-infected horses by complement fixation test, bacterial isolation, and PCR

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ABSTRACT

Glanders is an equine zoonosis caused by *Burkholderia mallei* that is responsible for considerable economic loss. Complement fixation testing (CFT) using warm or cold incubation are recommended by the OIE, but many routinely used detection tests may present misleading results. To increase accuracy of glanders diagnosis and establish an appropriate protocol in collaboration with the National Equine Health Program, seven horses positive for glanders kept in isolation in Brazil were examined fortnightly by CFT, microbiological screening, and molecular testing. Warm and cold serologies with USDA and c.c.Pro antigens, respectively, were performed on 132 samples using the US Department of Agriculture protocol. The warm and cold serologies showed, respectively,12.9% and 17.3% seroreactive, 85.7% and 65.2% non-reactive, 0.8% and 3% inconclusive, and 0% and 2.3% anticomplementary. The agreement of CFT protocols was moderate. Of 213 clinical samples submitted to selective culture (167 nasal swabs, 5 ocular swabs, 3 lymph node punctures, and 38 tissue samples from four horses that died), 1.9% tested positive for *B. mallei.* Fourteen samples and one nasal swab (7%) tested positive with PCR. Cold CFT with the USDA and c.c.Pro antigens, in combination with PCR to increase sensitivity, may be useful for diagnosis of chronic glanders.

1. Introduction

Glanders is a contagious infectious acute or chronic disease that affects mainly Equidae, but also humans, carnivores, and small ruminants. (Acha and Szyfres, 2003) The etiologic agent is *Burkholderia mallei*, a Gram-negative facultative intracellular bacillus (Khan et al., 2013).

The disease is notifiable to the World Organization for Animal Health (OIE). In Brazil, glanders is included in the National Equine Health Program (PNSE) coordinated by the Ministry of Agriculture, Livestock, and Supply (MAPA), the objective of which is to create strategies for epidemiological and zoosanitary surveillance and prophylaxis and eradication of diseases affecting equids (Brasil, 2018a; OIE, 2015). In case of seropositivity, horses must be euthanized (Brasil, 2018a).

The diagnosis of glanders can be established by clinical epidemiology; microscopy; complementary tests such as bacteria isolation, molecular identification, and the mallein test; as well as via serodiagnosis by the complement fixation test (CFT), ELISA, western blot, indirect immunofluorescence, and rose bengal staining (Naureen et al., 2007). This myriad of tests and heterogeneity of protocols have led to confusion and distrust of laboratory results, causing delay in the euthanasia of animals and risking the spread of the disease. These issues affect successful eradication of the disease in Brazil (Brasil, 2018b).

Complement fixation testing has been reported to show sensitivity of 90–95% in animals presenting clinical manifestations (Sprague et al., 2009; Khan et al., 2013). However, false-negative reactions are occasionally observed in the sera of young, pregnant, and older animals, and a false-positive reaction can occur in approximately 1% of the sera tested due to the use of crude total antigen of *B. mallei* generating cross-reactions with nonspecific antibodies present (Sprague et al., 2009; Khan et al., 2013). Intra-laboratory modifications to adapt the

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protocol to the antigen may compromise the quality of the assay, resulting in limitations in specificity and sensitivity. Some authors question the applicability of CFT due to the variation in reaction inputs, which may include hemolysin, complement, and sheep erythrocyte (Verma, 1990; Neubauer et al., 2005).

Complement fixation is the accepted serological test for the diagnosis of glanders in international trading of equids (OIE, 2015). False-positive reactions may cause financial loss, and false-negative tests may result in the introduction of glanders into healthy equine populations in previously glanders-free areas (OIE, 2015). Microbiological culture is the gold standard for glanders confirmation according to the OIE, but *B. mallei* presents unique culture characteristics, including the requirement for a glycerol-dependent culture medium and slow growth necessitating 72 hours incubation (OIE, 2015).

Isolating *B. mallei* from clinical specimens, mainly nasal swabs, presents a challenge due to high occurrence of other bacteria (Merwyn, Kumar, Agarwal and Rai, 2010). This is compounded by the low bacterial load usually present in infected equines, which generally do not show clinical manifestation of chronic disease (Scholz et al., 2006).

Polymerase chain reaction (PCR) can be an important tool for detecting *B. mallei*, since it has high sensitivity and does not depend on the viability of the agent for its identification (Merwyn et al., 2010). Detection of *B. mallei* by molecular methods is recommended for both acute and chronic manifestations, due to the rapid identification of the agent, differential diagnosis from melioidosis *Burkholderia pseudomallei*, and conclusiveness of the diagnosis (Suppiah, Thimma, Cheah and Vadivelu, 2009; OIE, 2015).

Government animal health programs face a challenge when dealing with herds in which individual animals represent high monetary value, as with horses. In these cases, a positive diagnosis must be as accurate as possible to avoid economic loss and reluctance of owners to participate in testing programs.

The present work aims to evaluate bacterial isolation and PCR and compare them with sero-diagnostic results obtained through cold and warm CFT protocols using USDA and c.c.Pro (Nederlands) antigens to test clinical samples obtained from horses confirmed positive for glanders related to outbreaks in Brazil. We expected to determine the most sensitive method and increase the reliability of glanders diagnosis in accordance with the PNSE and One Health Approach sanitary principles (Kahn, 2019).

2. Materials and methods

2.1. Animals and samples

The project was approved by the Ethical Commission on Animal Experimentation of the Biological Institute (CETEA-IB) on 7 December 2017 and registered under protocol number 156/17.

We obtained seven horses of both sexes, different breeds, and various ages from properties prohibited from equine trade by the Agricultural Defense Coordination of São Paulo State (CDA), the official veterinary service for suspected clinical cases of glanders. Horses had tested positive for glanders in a screening test by warm CFT, and diagnosis was confirmed by western blot performed in an official laboratory of in the PNSE, in accordance with Brazilian legislation (Brasil, 2004). Khan et al. (2011) have previously showed sensitivity and specificity of 62.2 and 96.5%, for warm CFT and Western blot respectively, and 100% was demonstrated for both in another study by Elschner et al. (2011). Usually glanders-positive animals are immediately euthanized after confirmation by CDA, but the seven were included in a larger clinical and epidemiological study in which they could be followed for a longer time. Considering a prevalence of the disease in Brazil of 43% (Teles et al., 2012) and using the lowest values for sensitivity and specificity of the two tests, we calculated a 96.4% predictive positive value for glanders, which is high enough for the analysis of the number of horses available.

The horses were held in the MAPA Quarantine Station in Cananéia, SP in a controlled biosafety environment isolated from the urban area. They were kept in separate stalls, each with a feeder, water fountain, and solarium with pasture supplemented with hay, commercial feed, and mineral salt according to nutritional requirements. Stalls were cleaned twice daily by trained caregivers who inspected the animals for general condition. From November of 2016 until February of 2018, during the collection of clinical samples, the animals were inspected for symptoms including lymph node enlargement, nasal or ocular secretion, limb edema, and lameness, and, when applicable, material was *collected for direct identification of the causative agent, according to an* established protocol (Khan et al., 2013).

2.2. Complement Fixation Test (CFT)

The CFT was performed at the Laboratory of General Bacteriology of the Biological Institute and fortnightly blood samples were obtained aseptically from all seven animals by venipuncture of the jugular into vacutainer tubes suitable for clot retraction. Blood was centrifuged, transferred to 1.5 mL microtubes, labelled for individual identification, and stored until analysis.

Serum samples were analyzed according to the protocol established by MAPA (Brasil, 2018a, 2018b) in accordance with the OIE (2015), using the United States Department of Agriculture (USDA) antigen, produced with the Chinese *B. mallei* strain of human origin, titer 1:200, and the c.c.Pro antigen (GmbH) from the Central Veterinary Institute of Wageningen University and Research (CIDC), consisting of the Mukteswar (India), Borgor (Indonesia), and Zagreb (Croatia) strains derived from horses, diluted 1:160. Using the USDA antigen, warm incubation was conducted in a bacteriological incubator at 37 °C for one hour and cold incubation in a refrigerator at 2–8 °C for 18 hours. With the c.c.Pro antigen, the cold method was used according to the manufacturer's instructions. Serial two-fold dilutions were performed on the CFT, 1:5 to 1:320. The interpretation was negative when 100% hemolysis was observed at 1:5 dilution, inconclusive with 25–75% hemolysis at the 1:5 dilution, and positive when no hemolysis was detected at 1:5 dilution (OIE, 2015).

2.3. Bacterial Isolation

When nasal and ocular secretions were observed during sampling, they were collected using sterile swabs. Lymph node puncture was conducted in animals with lymph node enlargement.

Three horses died during the monitored period and were necropsied. Samples of organs from the respiratory, circulatory, digestive, genitourinary, central nervous, mucosal, cutaneous, and lymphatic systems were collected. The protocol for collection and handling of samples was performed as described (Zachary, 2016).

Aliquots of clinical samples (nasal and ocular secretions, puncture of purulent abscesses of the ganglion chain, and organs for microbiological analysis) were stored in brain heart infusion (BHI) (2% glycerol) at 2–8 °C, and 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).

A suspension of each clinical sample for microbiological analysis was seeded on 5% sheep blood agar with 5% glycerin and 2500 IU potassium benzylpenicillin (selective medium for *B. mallei*) and incubated for 48–72 hours at 37°C (Quinn et al., 2005; Winn et al., 2008; Merwyn et al., 2010). Morphological characteristics of the resultant bacterial colonies were recorded, including size, shape, color, and presence and type of hemolysis. The colonies were examined by Gram staining microscopy for morphology, cell layout, and stain characteristics. *Burkholderia mallei* was identified by biochemical protocol assessing catalase, oxidase, indole, nitrate reduction, results of the Voges-Proskauer test, motility, and fermentation of sugars(Quinn et al., 2005; Winn et al., 2008).

2.4. Polymerase Chain Reaction (PCR)

For PCR analysis, we performed a ten-fold serial dilution of *B. mallei* on the MacFarland scale 1 (10^0) to 10^{-7} dilution. A 0.1 mL sample of the suspension was streaked onto 5% sheep blood agar supplemented with 2% glycerol, incubated at 37°C for 72 hours, and colony forming units (CFU) were counted. PCR detection threshold was obtained by dilution of *B. mallei,* all the dilutions were processed for DNA extraction in PCR assays cited in the following topics to determined the analytical sensitivities.

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, boiled for 10 minutes for bacterial inactivation. Organs from clinical samples (lung, liver, spleen, trachea, lymph nodes), lymph node punctures and swabs (nasal and ocular) were suspended in 0.85% saline sterile solution (1:5 w/v dilution). DNA extractions of all samples (colonies, organs, swabs and aspirateds) were performed from the supernatant using a commercial kit (Quick-gDNA MiniPrep reagent Zymo-Spin IIN columns, Zymo Research, Irvine, CA, USA). After DNA extraction, PCR was performed for detection of the flagellin (FliP) *B. mallei* gene using primers modified as previously described (Scholz et al., 2006) Bma-IS407-flip-F (5′TCAGGTTTGTATGCTCGG3′) and Bma--flip-(5′GCCCGACGAGCACCTGATT 3′) yielding a 528 bp fragment (Tomaso et al., 2006).

Amplification of all samples was performed using 10 μL of DNA plus 40 μL of PCR reagent mixture containing 1.25 U Taq DNA polymerase, 200 μM of each dNTP, buffer (10 mM Tris-HCl, pH 8.0, 50 mM KCl), 2 mM MgCl2, and 5 pmol/μL of each primer subjected to the following conditions: initial denaturation of 95°C for 4 minutes followed by 30 cycles of denaturation at 94°C for 1 minute, hybridization at 60°C for 1 minute, and extension at 72°C for 1 minute with a final extension of 72°C for 7 minutes. 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 plus 10000X Gelred staining solution (Biotium, USA) at 1:125. Visualization of the bands was performed with an ultraviolet light transducer.

2.5. Statistical Analyses

The Kappa index, expressing the correlation between the warm CFT and cold CFT, was obtained using the MINITAB version 16. (Thrusfield and Christley, 2018).

3. Results

The seven horses were monitored fortnightly, obtaining 26 collections that yielded 132 serum samples, 213 samples for bacterial isolation: 167 nasal swabs, 5 ocular swabs, 38 organ samples (lung, liver, spleen, trachea, tracheal lymph nodes, mediastinic lymph node), and 3 lymph node punctures from different animals.

3.1. Serology

The warm and cold CFT incubation found, respectively,17 and 39 of 132 serum samples (12.9% and 17.3%) seroreactive; 114 and 86 (85.7% and 65.2%) non-reactive; one and four (0.8% and 3%) inconclusive; and no and three samples (0% and 2.3%) anticomplementary. (Table 1)

To calculate the degree of agreement (Kappa Index) between warm and cold CFT, we considered reactive and non-reactive, excluding inconclusive and anticomplementary samples, for a total of 124 samples. The agreement between cold and warm CFT was considered moderate (Kappa 0.484 with error of 0.097and 95% CI from 0.294–0.674) (supplementary material).

3.2. Bacterial Isolation

A total of 160 (75.1%) of the samples showed growth of *Bacillus* spp., *Staphylococcus* spp., *Enterobacteriacea,* and non-fermenting negative

Table 1
Results of serological testing of samples from seven horses, analyzed with warm and cold complement fixation test (CFT), collected every 14 days (26 samplings) over 59 weeks during 2016-2018, in Brazil.

Horse	1		2		3		4		5		6		7	
Week	CFT Warm	Cold	CFT Warm	Cold	CFT Warm	Cold	CFT Warm	Cold	CFT Warm	Cold	CFT Warm	Cold	CFT Warm	Cold
1	R	R	NR	R	NR	R	NR	NR	NR	R	NR	NR	NR	NR
3	R	R	NR	NR	NR	NR	b	b	NR	AC	NR	NR	NR	NR
5	R	R	NR	R	NR	NR	-	-	NR	NR	NR	NR	NR	NR
7	NR	R	NR	R	NR	NR	-	-	NR	NR	NR	NR	NR	NR
9	NR	NR	NR	R	NR	R	-	-	NR	NR	NR	NR	NR	NR
11	R	R	NR	IC	NR	NR	-	-	NR	NR	R	R	NR	NR
13	NR	NR	NR	R	NR	NR	-	-	NR	NR	NR	NR	NR	NR
15	NR	R	a	a	NR	NR	-	-	NR	R	NR	R	NR	NR
17	R	R	-	-	NR	NR	-	-	NR	R	NR	NR	NR	NR
19	NR	NR	-	-	NR	NR	-	-	NR	NR	R	R	NR	NR
22	NR	R	-	-	NR	R	-	-	NR	NR	NR	R	NR	NR
25	R	R	-	-	NR	NR	-	-	NR	NR	NR	NR	NR	NR
27	NR	R	-	-	NR	NR	-	-	NR	NR	NR	NR	NR	NR
30	R	R	-	-	NR	NR	-	-	NR	NR	NR	R	NR	NR
33	R	R	-	-	NR	NR	-	-	NR	R	IC	R	R	R
37	R	R	-	-	NR	NR	-	-	NR	NR	NR	NR	R	R
39	R	NR	-	-	NR	NR	-	-	NR	NR	NR	NR	R	R
41	R	R	-	-	NR	NR	-	-	NR	NR	NR	NR	NR	AC
43	R	R	-	-	NR	NR	-	-	NR	NR	NR	NR	NR	NR
45	NR	NR	-	-	NR	NR	-	-	NR	NR	NR	NR	NR	NR
47	NR	IC	-	-	NR	R	-	-	NR	AC	NR	NR	NR	NR
49	a	a	-	-	NR	NR	-	-	NR	NR	NR	NR	NR	IC
53	-	-	-	-	NR	R	-	-	NR	NR	NR	NR	NR	NR
55	-	-	-	-	NR	R	-	-	NR	NR	NR	NR	NR	IC
57	-	-	-	-	NR	NR	-	-	NR	NR	NR	NR	NR	NR
59	-	-	-	-	NR	NR	-	-	NR	NR	NR	NR	NR	NR

R = reactive; NR = non-reactive; IC = inconclusive; AC = anticomplementary a= Death of the animal; b= Animal euthanized.

bacilli (contaminants). Four colonies (1.8%) were confirmed as *Burkholderia* spp., and 49 samples (23%) showed no growth after microbiological culture.

3.3. DNA detection

The detection limits was determined by agarose gel electrophoresis (1%) of the DNA products from bacterial dilutions. We were able to detect dilution of 10^{-6} UFC/mL in the gel and using the bacterial counting method, threshold was established as $1{,}3 \times 10^{1}$ UFC/mL. Using the spectrophotometer (Spectrum Pharmacy), absorbance of 280 nm, we observed that this detection limit was equivalent to 20 ng of bacterial DNA.

We observed 15/213 samples (7%) positive by PCR (Table 2) from the three necropsied horses.

4. Discussion

The use of serological approaches only for glanders diagnosis is a primary challenge in Brazil. We studied naturally infected horses with the CFT results classified as reactive, inconclusive, or anti-complementary and established better diagnostic tools when conducted in combination with microbiological and molecular tests in an endemic situation.

A previous study (Silva et al., 2009) obtained positive *B. mallei* isolation in eight samples of closed skin nodules from equines used to pull carts in sugar cane mills in the Zona da Mata region of Pernambuco and Alagoas States, Brazil. The stress of this type of work can make animals vulnerable to development of glanders in its acute form. The animals showed respiratory signs with mucopurulent nasal secretion, nasal mucosa ulcers, and star-shaped scars on the nasal mucosa, as well as weakness, superficial lymph node enlargement, lack of appetite, and limb edema (Silva et al., 2009). We were able to isolate *B. mallei* by means of methods previously described (Merwyn et al., 2010), but from a smaller proportion of tested samples, possibly because the quarantined horses did not present overt symptoms characteristic of glanders, indicating that they presented the chronic form of the disease, reducing the chance of isolating the bacteria. However, we obtained positive isolation in a horse that was euthanized after showing clinical symptoms, despite its being non-reactive by warm and cold CFT. We hypothesize this to be due to an intermediate status between acute and chronic glanders. A previous study showed that cold CFT presented lower specificity compared with other serological tests (Elschner et al. 2019), which may explain the false negative result from the horse in the present study. The horses #1 and 2 were negative for bacterial growth, but were positive in the PCR and showed at least one positive result for FC.

Using PCR, *B. mallei* was detected in several samples with characteristic organ lesions: multiple lung nodules, microscopic liver abscesses, and small spleen scars. No nasal or ocular swabs of animals positive for *B. mallei* by CFT were positive by isolation or PCR, with the exception of one collected from the deep nasal cavity during necropsy, an area that would not be accessible for regular sampling. This secretion of the respiratory agent may be present only in animals with acute disease, when transmission is largely via the respiratory tract. Authors reported difficulty in isolating *B. mallei* from clinical specimens, mainly nasal swabs, due to high presence of other bacteria (Merwyn et al., 2010).The OIE states that, in animals with chronic disease, isolation and biochemical identification is a challenge, even with the use of antibiotics to inhibit secondary contamination (OIE, 2015). It was reported that bacterial isolation from nasal swabs is problematic in animals with chronic and subclinical diseases due to the low number of viable bacteria in the samples and high presence of local bacterial flora (Scholz et al., 2006). We did not isolate *B. mallei* from this site when using a glycerol-enriched culture medium with antibiotics to inhibit secondary results. A previous study reported isolation of *B. mallei* in samples incubated in BHI broth with 3000 IU of benzyl penicillin/mL for three hours at room temperature prior to being seeded on 5% sheep blood agar with 4% glycerol and incubated at 37 °C for 48 hours under aerobic conditions (Khan et al., 2011).

In the present study, we detected a lower PCR threshold (10^{1} CFU/mL) when comparing with previous results (10^{2}–10^{3} CFU/mL) that standardized PCR for detection of *B. mallei* and *Burkholderia pseudomallei* with the FliC and the 23S rRNA gene (Altukhova et al., 2007). Another study (Lee, Wang and Yap, 2005) that conducted multiplex PCR using *B. mallei* strains isolated from human, horse, and mule clinical specimens, obtained a detection threshold of 100 pg DNA, equivalent to 20, 000 genomes. Therefore, our protocol showed better analytical sensitivity. A hypothesis for the difference in tests performances could be the interval between collection of the samples and laboratorial analysis. From previous experience, we observed that the faster the samples were processed, with minimal interval after they were obtained from the animals, the quality of the DNA products was improved.

A prior study (Khan et al., 2011) tested 410 serum samples (342 horses, 7 donkeys, 30 mules, 12 rabbits, and 19 horses immunized against *B. mallei*) using cold CFT with c.c.Pro, the Central Veterinary Institute of Wageningen URCIDC, and USDA antigens and found the USDA antigen to produce 100% specificity compared to 97.5% for the CIDC antigen and 96.5% for the c.c.Pro. In agreement with this broad study, we found cold CFT with USDA and c.c.Pro to be superior to warm CFT with the USDA antigen; hence, cold CFT is appropriate for glanders screening in Brazil. Although bacterial isolation is considered the gold standard for glanders diagnosis, it is necessary to perform complementary tests such as PCR, since PCR is more sensitive than isolation independent of the viability of the agent and can detect *B. mallei* even in a sample with secondary contamination.

In summary, we found cold CFT be more appropriate to our conditions than warm and suggest it as a tool to be used in disease eradication programs, based on its ability to better detect positive animals, which can be subsequently confirmed with complementary tests. Polymerase chain reaction is an important method for the detection of bacteria in tissues of infected animals, especially in chronic disease, and may be complementary to the serological tests used in control and eradication programs. These data contribute to clarifying gaps related to laboratory testing for glanders and aid in decision-making in Brazil PNSE.

Table 2
Clinical samples from tissues of horses with glanders assayed by polymerase chain reaction (PCR) and microbiological culture.

Horse	Tissues	Bacterial Growth	PCR
1	Heart	-	+
	Tracheal LN	-	+
	Kidney	-	+
2	Skin abscess	-	+
	Lung	-	+
	Nasal swab at necropsy	-	+
4	Mesenteric LN	-	+
	Lung	+	+
	Submandibular LN	+	+
	Nasal cavity lesion	+	+
	Pharyngeal LN	-	+
	Mediastinic Ln	+	+
	Tracheal LN	-	+
	Trachea lesion	-	+
	Trachea secretion	-	+

Ethical statement

We are submitting the paper entitled “Systematic monitoring of glanders-infected horses by complement fixation test, bacterial isolation, and PCR “ for analysis by the editorial boarding of the Veterinary and Animal Science. We declare that this research was approved by the Ethics Committee on Animal Experiments of the Biological Institute (CETEA-IB) on 7 December 2017, registration number CETEA 156/17.

Conlilct of interest

We are submitting the paper entitled “Systematic monitoring of glanders-infected horses by complement fixation test, bacterial isolation, and PCR “ for analysis by the editorial boarding of the Veterinary and Animal Science. We declare that there is no financial or personal relationship that could cause a conflict of interest regarding this article.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.vas.2020.100147.

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