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REPORT ON THE PERFORMANCE OF THE ELISA-BKM16 TEST

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ABSTRACT

Glanders is an infectious disease of solipeds and humans caused by the Gram-negative bacillus *Burkholderia mallei*. It requires immediate notification of any suspect case in Brazil and confirmed cases should be immediately reported to the OIE.

This work provides information on the performance of the ELISA recently created by PANAFTOSA-PAHO/WHO using a recombinant protein (known as ELISA-BKM16), compared to complement fixation (CF) and Western Blot (also based on the same recombinant protein) tests, carried out in three different studies: one using a known panel (56 serums); another with a longitudinal follow-up in a controlled environment (in 21 horses), and the last one using samples obtained from surveillance (377 samples).

The results of the investigations using a known panel achieved a sensitivity and a specificity of 1. The longitudinal study in isolated animals shows (as expected) good agreement between the two tests (ELISA-BKM16 and WB), while in CF a longitudinal variation is observed alternating between positives and negatives, and showing anti-complementary (AC) results. Moreover, an euthanized animal, confirmed as positive by PCR, showed consistent positive results to ELISA and WB in the months prior to death, but negative to CF.

Furthermore, in samples from movement control, ELISA-BKM16 shows very good agreement compared to WB, reaching a sensitivity of 1 while maintaining a high specificity (0.99). Nevertheless, compared to WB, CF sensitivity was 0, with a specificity of 0.99. According to the results of this study, ELISA BKM16 emerges as a potential alternative to CF for glanders surveillance and control, providing good operating characteristics (sensitivity and specificity) in addition to cost-effective advantages due to its easy implementation at the laboratory.

Keywords: *Glanders, ELISA, Complement fixation, Western Blot, Surveillance*

1. INTRODUCTION AND CONTEXT

Glanders is an infectious disease of solipeds and humans caused by the gram-negative bacillus *Burkholderia mallei* (Khan et al., 2013). It is one of the oldest known diseases of the horse and a contagious, sometimes fatal, zoonotic disease (Van Zandt et al., 2013).

According to the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals of the OIE, chapter on glanders updated in 2015, the complement fixation (CF) test is an accurate and reliable serological method for the diagnosis of glanders and it is considered highly sensitive (OIE, 2017a). However, a recent study showed that the sensitivity of this method can be affected by the protocol used with the risk of misclassification in case of low CF titers (Laroucau *et al.,* 2016), most probably in chronically infected animals. The risk of false-negatives and even false-positives was also mentioned by Malik (2016). On the other hand, the Western Blot (WB) test is presented as an alternative in eradication policies and prevalence studies (OIE, 2017a). This test was considered highly specific in a study using a crude non-recombinant protein (Elschner *et al.,* 2011). Besides, WB has been considered an alternative to CF in endemic countries (Elschner *et al.,* 2011).

Different ELISA formats -competitive and indirect formats, among others- using purified or recombinant fractions of crude *B. Mallei* have been developed to aid in the diagnosis of glanders, since the use of different antigens has a direct effect on the specificity of the test. According to Singha *et al.* (2014), the tests developed using the TssB protein have shown a high sensitivity of about 99,7% and a specificity of 100%. The use of recombinant TssB protein has the advantage of no cross-reactivity with *melioidosis*, being highly specific for *B. mallei* antibodies (Malik, 2016; Singha *et al.*, 2014); that is, it can differentiate anti-*B. mallei* from anti-*B. pseudomallei* antibodies. Hence, a specific serological test able to detect anti-*B. mallei* antibodies without cross-reactivity with other bacteria from the Bulkholderia genus is highly desirable. This characteristic in ELISA development (also applicable to WB) offers advantages over complement fixation, in addition to an easy and quick implementation and a better cost-benefit ratio.

Although glanders has been eradicated in several countries, it has gained the re-emerging status due to several recent outbreaks (Khan *et al.*, 2013). Specifically, the disease has recently become of high interest for veterinary services in South America due to an apparent re-emergence, particularly in Brazil, where several outbreaks have been detected in recent years. For this reason, glanders is included in the list of immediately notifiable diseases in the event of any suspect case in Brazil for the application of Animal Health Protection measures, and confirmed cases are to be reported to the OIE (Brazil, 2018, 2004a, OIE, 2017b, 2017c; Van Zandt *et al*., 2013).

All the above has reopened the debate on the best surveillance strategy and the diagnostic tools used, particularly as regards confirmation of a diagnosis of glanders. At present, the CF method recommended by Manual of Diagnostic Tests and Vaccines for Terrestrial Animals of the OIE for confirmation of clinical cases, prevalence studies and eradication programs shows inconsistent results. This is thought to occur due to the epidemiological situation in Brazil, where disease occurrence is low and in most cases horses are chronically infected and, therefore, the concentration of complement-fixing anti-*B. mallei* antibodies is low. Further studies on the pathogenesis of the disease on equine species are needed to better explain this hypothesis.

In an attempt to improve the performance characteristics of the test, PANAFTOSA-PAHO/WHO developed the ELISA-BKM16 using a recombinant *B. mallei* TssB protein; whose name comes from the first and fourth letters of "Burkholderia" along with the number that expresses the year in which it was developed in 2016). Alongside, PANAFTOSA-PAHO/WHO developed a WB, also using a recombinant *B. mallei* TssB protein.

2. OBJETIVE

This work is aimed at understanding the performance of the recently developed ELISA-BKM16 by comparing it with other existing tests such as the complement fixation (CF) test currently used for surveillance in Brazil, and the Western Blot (WB) test, considered the reference alternative to the CF. This work intends to be a tool to understand the current options for the diagnosis of glanders during routine surveillance and control in Brazil and other South American countries.

3. MATERIALS AND METHODS

3.1 Strategy

The strategy to assess ELISA-BKM16 performance includes three components:

- For the first component, a direct validation is carried out checking the results of the ELISA-BKM16 against a reference panel used in Brazilian official laboratories.
- For the second component, samples of horses kept in isolation in the Quarantine Station of Cananéia and longitudinally monitored with blood samples collected every 15 days were used. The animals provided for this research were selected according to positive (or inconclusive) CF results in transit control.
- For the third component, the results of ELISA-BKM16 and CF were compared to those of WB (taken as the reference test in this case), using samples of horse transit control. It should be highlighted that comparisons between ELISA-BKM16 and WB are essentially a comparison between two methodologies, assuming that the WB technique offers better diagnostic characteristics, since both tests are based on the same recombinant antigen.

3.2 Methodology for ELISA-BKM16 validation with a known panel

3.2.1 Description of the test used

The ELISA-BKM16 kit is designed to detect antibodies against *B. mallei* in horse sera, thus determining the presence of glanders infection. The kit enables to carry out an indirect ELISA assay in which a recombinant antigen (of TssB protein) specific for *B. mallei* species is fixed in a 96-well microplate. In the presence of a serum containing bacterium-specific antibodies, an antigen-antibody complex is formed and non-specific antibodies are removed from the plate after the washing stage. The resulting complex is recognized by a second anti-horse IgG antibody conjugated with peroxidase. Once added to the substrate containing H2O2, this enzyme is bound to the substrate (chromogen) leading to the formation of yellowish complexes, which indicate that antibodies against *B. mallei* have been found. In order to standardize results, the following formula was used: (OD sample –mean OD NC)/ (mean

OD PC–mean OD NC) *100. Where OD is the optical density obtained in the test, NC is the negative control, and PC is the positive control.

3.2.2 Description of the panel and data source

For this task, a panel provided by the National Agriculture Laboratory (LANAGRO), located in Pernambuco, was used. This panel of 56 serums (consisting of horse, mule and donkey sera) was produced from positive and negative samples established according to current Brazilian regulations (Brazil, 2004a, 2004b).

3.2.3 Statistical analyses

The sensitivity and specificity (Banoo *et al.,* 2008) of the ELISA-BKM16 test were calculated and compared to the LANAGRO reference panel, together with the 95% confidence intervals, assuming that data are obtained by binomial sampling. For the ELISA, different cut-off points of standardized results were used in order to identify the one yielding the best performance.

3.3 Methodology used to compare ELISA-BKM16, CF and WB performance in horses examined every two weeks.

3.3.1 Description of tests used

The CF test was performed compliant to the protocol established by the MAPA (BRAZIL, 2018) in agreement with the technique described (OIE, 2017a), using control sera and antigens registered in the MAPA or imported with MAPA's authorization, following the manufacturer's instructions for the incubation period. Base 2 serial dilutions were made in the test, starting with an initial dilution of 1:5 to 1:320. Tests were interpreted as follows: NEGATIVE when presenting 100% hemolysis in the 1:5 dilution, INCONCLUSIVE (suspect), when 25 to 75% hemolysis remained in the 1:5 dilution, POSITIVE, 100% free from hemolysis in the 1:5 dilution (Brazil, 2004b; OIE, 2017a).

In this case, the WB is a sensitive enzyme immunoassay consisting of a specific recombinant antigen of the *B. mallei* species fixed to nitrocellulose strips, the antigen was the same used in the ELISA-BKM16. In the presence of serum containing disease-specific antibodies, an antigen-antibody complex is formed, which is recognized by a second antibody directed towards immunoglobulins of the species under investigation and, in turn, labeled with alkaline phosphatase (anti-horse IgG conjugated with alkaline phosphatase). Once the suitable substrate for the enzyme is added, a purplish strip appears indicating that anti-*B. mallei* antibodies have been identified.

3.3.2 Description of sample source

Using the results of the CF assay in the official control of animal transit, 21 positive and inconclusive horses of both sexes and different breeds and ages were donated for the investigation, offering an opportunity for a longitudinal follow-up. The animals were kept in isolation at the MAPA's Quarantine Station in Cananéia-SP from July 2015 to August 2017, and both blood and nasal and ocular secretion

samples were collected every two weeks. It should be highlighted that MAPA's Quarantine Station offers a controlled and biosafe environment, is completely isolated from the urban area, and has an adequate structure for keeping animals with infectious diseases. Until August 2017, 1,400 serum samples of these animals were obtained. Sera were analyzed by CF, ELISA-BKM16 (applying a cut-off point of 20), WB for anti-*B.mallei* antibody detection, and PCR for the presence of B. mallei in nasal and ocular secretion samples (OIE, 2017a).

A focus was defined as the epidemiological unit where at least one case of glanders was confirmed by the Official Veterinary Service. All the horses taken to Cananéia were considered animals coming from a focus and were submitted to clinical and epidemiological examination, including suspect cases and other equines in the facility. Differences were observed in the investigated equines; clinical manifestations at presentation were evident in certain cases while not in others since, at the time of movement, equines were already in the chronic stage of the disease. Therefore, Cananéia received animals with "no negative" results to the CF performed by the official control (i.e., positive and inconclusive results, and others showing anti-complementary activity). Until November 2016, Cananéia had only chronically infected and negative equines (confirmed by ELISA and WB). In November that year, six equines coming from a focus in Capivari were admitted, which had been monitored by the study team in the six months prior to entry and initially considered clinical cases. In addition to the official CF test, these animals were tested by CF, ELISA and WB by the study team at the time of official detection, and they were monitored for six months prior to transport to Cananéia.

This study was approved by the ethics commission on animal experimentation (CETEA-IB) in July 2015. This certifies that the protocol Nº 142/15 described in this study complies with the ethical principles on animal experimentation adopted by the Brazilian Association for Laboratory Animal Science (SBCAL/COBEA), by el National Council of Animal Experimentation Control (CONCEA) and the Brazilian Guidelines for the Care and Use of Animals for Scientific and Didactic Purposes (DBCA).

3.3.3 Descriptive Analysis

Animals were longitudinally evaluated according to sample results and clinical manifestations. According to the longitudinal interpretation of these results, animals were classified as: negative) no clinical manifestations and no reaction to any ELISA, WB and CF tests; chronically positive) no apparent clinical manifestations, but reactive to most ELISA and WB tests and negative to CF (with some exceptions in the latter due to fluctuations in test results); and acutely positive) clinical manifestations typical of glanders and reactive to the three tests (with some exceptions in CF due to fluctuations in test results).

3.4 Methodology to compare ELISA-BKM16 and CF performance in movement control surveillance compared to WB

3.4.1 Description of the reference standard test used

The WB test was used as reference for this comparison. The development of the WB test used has been described above.

3.4.2 Description of the tests to be compared

The development of the CF and ELISA-BKM16 tests has already been described in this document.

3.4.3 Description of the panel and data source

This task was performed using a panel consisting of 377 serum samples analyzed for glanders by CF, obtained from Paddock Laboratories of São Paulo-Brazil (282 samples) and the Biological Institute (95 samples), both accredited by the General Accreditation Coordination - (Cgcre) – Inmetro, and accredited and certified by the Ministry of Agriculture, Livestock and Supply (MAPA) to carry out assays and provide results according to official programs and controls. Blood of horses intended for movement from the states of RJ (2) MG (17), MS (4), SC (53), GO (6), RS (67), PR (28), SP (200) was collected between 04/10/2017 and 05/22/2017. The samples were analyzed by the complement fixation test within 24 hours after arrival at the laboratory.

3.4.4 Statistical analyses

Sensitivity and specificity of CF and ELISA-BKM16 tests, together with the 95% confidence intervals, were calculated and compared with the WB used (Banoo et al., 2008), assuming that data are obtained by binomial sampling. Different cut-off points were calculated for ELISA in order to determine which one offered the best performance. In addition, the receiver operating characteristic (ROC) curve was calculated to make a graphical assessment of ELISA performance at different cut-off points (Sing *et al.,* 2005). Although this exercise is not intended as a validation in itself but rather a comparison to WB performance, it was decided to use sensitivity and specificity measures instead of other agreement measures (such as Kappa). These measures (sensitivity and specificity) provide more information for performance interpretation in relation to the study target, for instance, to understand the presence of probable false negatives and false positives.

All the above mentioned analyses were conducted with R version R 2.5.1 (R Core Team, 2016), “bdpv” and “ROCR” packages.

4. RESULTS

4.1 Results for the validation of ELISA-BKM16 with a known panel

4.1.1 Description of the known panel results

This panel consists of 22 negative and 34 positive samples in agreement with the official legislation (Brasil, 2004a, 2004b). Distribution of standardized ELISA-BKM16 values is between -39.6 and 472.1, with a mean value of 160.6 (see Figure 1).

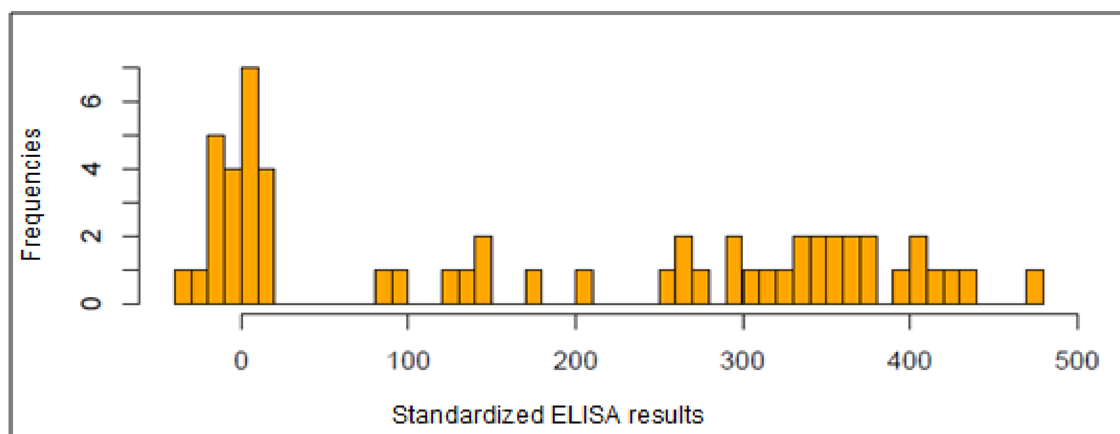

FIGURE 1. Histogram representation with frequency distribution of different ELISA-BKM16 results with a known panel of 56 samples.

4.1.2 Results of ELISA-BKM16 performance compared to the panel

For the different ELISA cut-off points tested (between 0 and 45), shown on Table 1, sensitivity remains unchanged, with a value of 1. However, specificity increases progressively until a cut-off point of 20, from which the specificity attained is 1.

TABLE 1. Results of sensitivity (Se) and specificity (Sp) calculations, comparing ELISA (10 different cut-off points) with a reference LANAGRO panel. N = 56.

Cut-off	Se	95% CI	Sp	95% CI
0	1	0.9-1	0.5	0.28-0.72
5	1	0.9-1	0.68	0.45-0.86
10	1	0.9-1	0.73	0.5-0.89
15	1	0.9-1	0.86	0.65-0.97
20	**1**	**0.9-1**	**1**	**0.85-1**
25	1	0.9-1	1	0.85-1
30	1	0.9-1	1	0.85-1
35	1	0.9-1	1	0.85-1
40	1	0.9-1	1	0.85-1
45	1	0.9-1	1	0.85-1

As seen in the graph representing the (kernel) density function of ELISA results for negative and positive samples (Figure 2), these subpopulations are highly differentiated, with negative sample results showing very low values in the ELISA, and positive results with higher values.

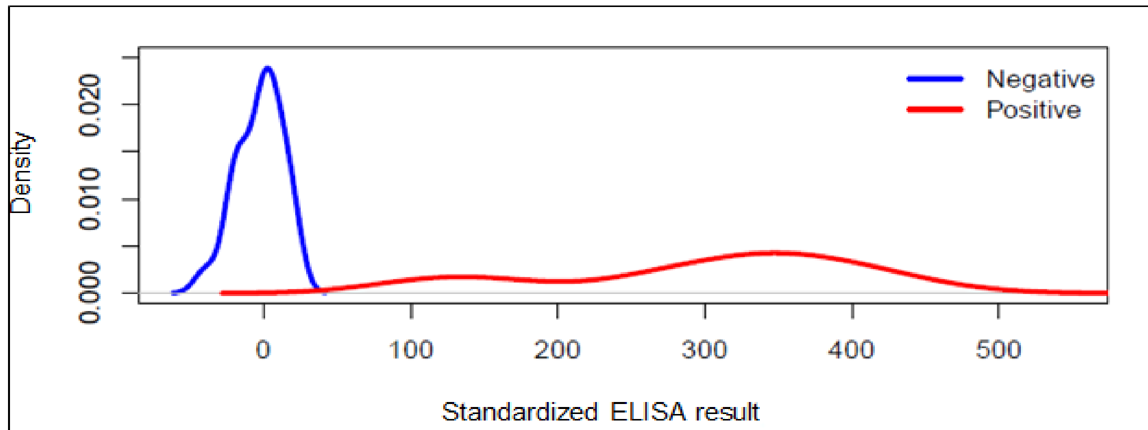

FIGURE 2. Kernel density function of ELISA-BKM16 results for negative and positive samples with a known panel of 56 samples.

4.2 Results for ELISA-BKM16, CF and WB performance comparison in horses inspected every two weeks

According to the longitudinal results presented on Table 2 and Figure 3, animals a, c, d, e, g, o, q, r, t, u were considered true negatives, animals b, f, h, p, s chronic positives, and animals i, j, k, l, m, n clinical positives. The latter were the animals from the Capivari focus which were monitored by the study team during the six months prior to transport to Cananéia. In the acute stage of the disease, these animals presented the typical clinical manifestation of the disease and tested positive in the three assays (CF, ELISA and WB). The six animals that survived the focus became chronically infected, negative in CF, and remained positive in ELISA and WB, a profile that was maintained during the whole monitored period (before and after transport to Cananéia).

PCR results from nasal swabs periodically taken from all animals were negative (as expected in chronic or negative ill animals at the Cananéia station)(OIE, 2017a). Clear longitudinal consistency and agreement between WB and ELISA results is observed both in negative and positive horses, except for the "s" horse (an intact male), in which ELISA result varies over time; on the other hand, CF results present a longitudinal variation between positive and negative, including the emergence of anti-complementary (AC) results, which does not allow to obtain a result for this test.

Animal "h" is detected as positive in ELISA and WB as of day 240, while it remains negative in CF at that time. This animal is thought to have had a seroconversion after a long incubation period (at least 8 months), or a reactivation. The likelihood of becoming infected at the quarantine station is low since the bacterium was not detected in any nasal swabs.

Animal “n” was the only one which had a post mortem examination results (after being euthanized). Necropsy was performed and anatomopathological findings were consistent with glanders: purulent pneumonia with multiple abscesses and granulomas, associated with Langhans giant cells. Purulent hepatitis. Moderate, non purulent membranoproliferative glomerulonephritis. Splenic hemosiderosis (reflecting a potential anemia). Histopathological lesions observed by hematoxylin-eosin staining were consistent with glanders and a PCR positive result was obtained in the purulent discharge found in the trachea. As observed in Figure 3, this animal was positive by WB and ELISA during the monitoring period in Cananéia, plus the additional six months prior to transport to the station, while during the period in Cananéia, CF results were negative.

TABLE 2. Summary information of horses and categorization results according to longitudinal results.

ID´s animal	Gender	Start-up date of tracking system / monitoring period	Classification according to longitudinal results
a	Female	jun/15	True negative
b	Female	jun/15	Positive chronic
c	Female	jun/15	True negative
d	Female	jun/15	True negative
e	Castrated male	jun/15	True negative
f	Castrated male	jun/15	Positive chronic
g	Castrated male	jun/15	True negative
h	Castrated male	jul/15	Positive chronic
i[¥]	Female	nov/16	Positive clinical
j[¥]	Female	nov/16	Positive clinical
k[¥]	Young male	nov/16	Positive clinical
l[¥]	Young female	nov/16	Positive clinical
m[¥]	Young	nov/16	Positive clinical
n[¥€]	Female	nov/16	Positive clinical
o	Castrated male	jun/15	True negative
p	Female	jun/15	Positive chronic
q[Ω]	Castrated male	jul/15	True negative
r	Female	jul/15	True negative
s	Entire male	jul/15	Positive chronic
t	Female	jun/15	True negative
u	Castrated male	jun/15	True negative

¥ Animals coming from the Capivari outbreak monitored from the 6 months prior to entry at Cananéia station
Animal n€ was euthanized and necropsied, and disease was confirmed by PCR and histopathology.
Animal qΩ was a maleinized animal

TABLE 3 presents ELISA, WB and CF results of a biweekly longitudinal study from the entry of animals at Cananéia for the detection of anti-B. mallei antibodies. For animals coming from the Capivari outbreak, the results from the monthly longitudinal follow up prior to Cananéia transport are also included.

ID	Test	Research days prior to Cananeia						Research days at Cananeia																											
		-180	-150	-120	-90	-60	-30	0	15	30	45	60	75	90	105	120	135	150	165	180	195	210	225	240	255	270	285	300	315	330	345	360	375	390	405
a	ELISA							NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
	WB							NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
	FC							NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	POS	POS	POS	POS	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
b	ELISA							POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS
	WB							POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS
	FC							NEG	NEG	NEG	NEG	NEG	NEG	NEG	AC	AC	NEG	AC	AC	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
c	ELISA							NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
	WB							NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
	FC							NEG	NEG	NEG	NEG	POS	POS	NEG	NEG	NEG	NEG	NEG	NEG	NEG	POS	NEG	NEG	AC	AC	POS	AC	AC	NEG	NEG	POS	AC	NEG	NEG	NEG
d	ELISA							NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
	WB							NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
	FC							POS	POS	POS	AC	NEG	AC	AC	AC	NEG	NEG	AC	NEG	POS	POS	NEG	POS	POS	POS	POS	POS	AC	AC	NEG	NEG	NEG	NEG	NEG	NEG
e	ELISA							NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
	WB							NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
	FC							NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	AC	AC	AC	POS	POS	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	POS	NEG	NEG	NEG
f	ELISA							POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS
	WB							POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS
	FC							NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	AC	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	POS	NEG	NEG	NEG	NEG
g	ELISA							NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
	WB							NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
	FC							POS	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	POS	NEG	NEG	NEG
h	ELISA							NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS
	WB							NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS
	FC							NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	POS	NEG	NEG	NEG	AC	AC	AC	AC	POS	NEG	NEG
i	ELISA	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS																	
	WB	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS																	
	FC	POS	NEG	NEG	NEG	POS	POS	NEG	NEG	NEG	POS	POS	POS	POS	POS	POS	NEG	POS																	
j	ELISA	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS																	
	WB	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS																	
	FC	POS	NEG	NEG	NEG	NEG	NEG	NEG	NEG	POS	NEG	NEG	POS	NEG	NEG	NEG	NEG	NEG																	
k	ELISA	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS																	
	WB	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS																	
	FC	NEG	POS	POS	NEG	NEG	NEG	NEG	NEG	POS	POS	NEG	NEG	NEG	NEG	POS	NEG	NEG																	
l	ELISA	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG																	
	WB	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG																	
	FC	NEG	NEG	NEG	NEG	NEG	NEG	POS	POS	POS	NEG	NEG	NEG	NEG	POS	POS	NEG	NEG																	
m	ELISA	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS																	
	WB	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS																	
	FC	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	POS	NEG	NEG	NEG	POS	POS	POS																	
n	ELISA	POS	POS	POS	POS	POS	POS	POS																											
	WB	POS	POS	POS	POS	POS	POS	POS																											
	FC	POS	NEG	NEG	NEG	NEG	NEG	NEG																											
o	ELISA							NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
	WB							NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
	FC							AC	AC	AC	AC	NEG	AC	NEG	AC	NEG	NEG	AC	NEG	NEG	NEG	NEG	NEG	NEG	AC	NEG	NEG	NEG	NEG	NEG	NEG	AC	NEG	NEG	NEG
p	ELISA							POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS
	WB							POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS
	FC							POS	POS	POS	POS	POS	POS	POS	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	POS	POS	NEG	NEG	POS	NEG	NEG	POS	NEG
q	ELISA							NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
	WB							NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
	FC							POS	POS	POS	POS	POS	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	POS	NEG	POS	NEG	NEG	NEG
r	ELISA							NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
	WB							NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
	FC							POS	POS	POS	NEG	NEG	NEG	NEG	NEG	NEG	POS	POS	NEG	NEG	NEG	NEG	NEG	POS	POS	POS	NEG	POS	NEG	NEG	NEG	NEG	NEG	NEG	NEG
s	ELISA							NEG	NEG	NEG	POS	POS	POS	POS	POS	NEG	NEG	NEG	NEG	NEG	NEG	POS	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
	WB							POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS
	FC							NEG	NEG	NEG	NEG	POS	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	POS	NEG	NEG	NEG
t	ELISA							NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
	WB							NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
	FC							NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	POS	NEG	NEG	NEG	NEG	POS	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	POS	NEG	NEG
u	ELISA							NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
	WB							NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
	FC							POS	NEG	NEG	NEG	POS	NEG	NEG	AC	POS	AC	AC	NEG	NEG	POS	POS	NEG	AC	POS	NEG	POS	POS	NEG	NEG	POS	AC	NEG	NEG	NEG

4.3 Results for the comparison of ELISA-BKM16 and CF performance in movement control surveillance

4.3.1 Description of panel results

WB results showed 365 negative and 12 positive samples. There were 374 negative and 3 positive samples by CF. Standardized ELISA results ranged between -19.46 and 165.1, with a mean value of -2.23. The histogram is shown in Figure 3.

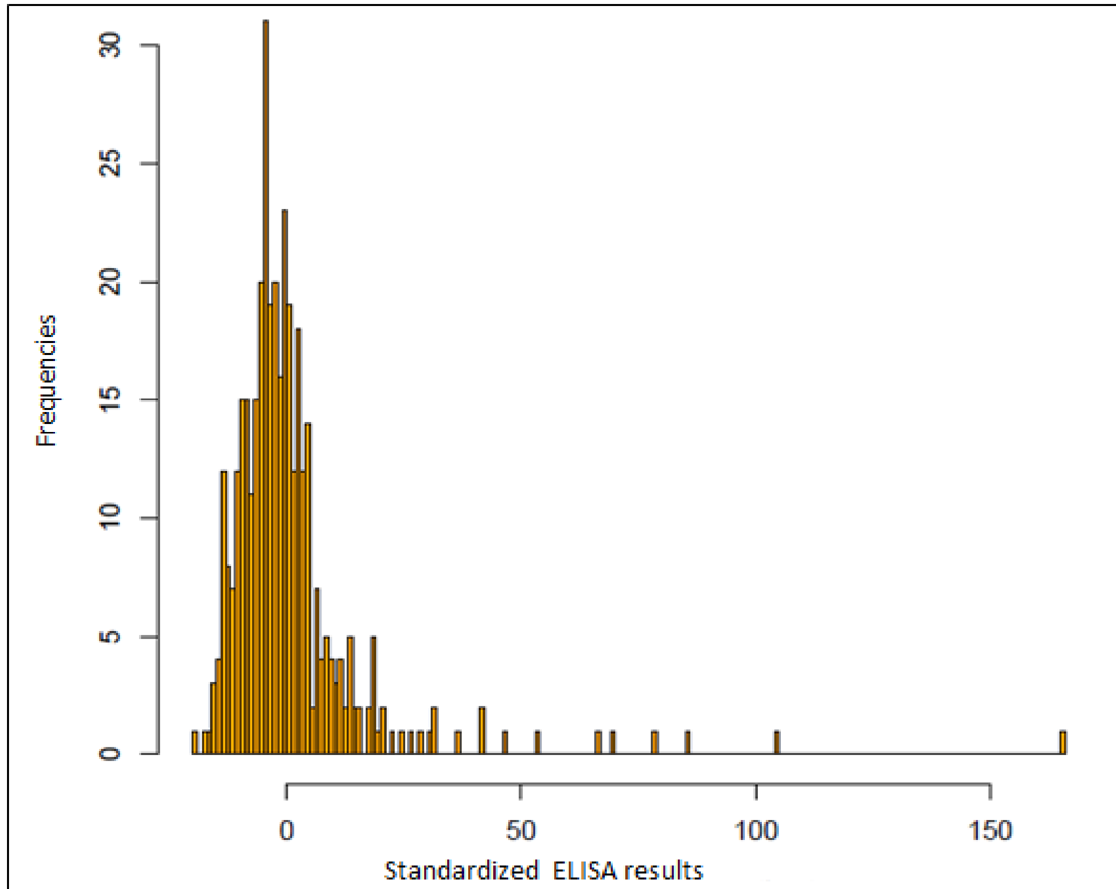

FIGURE 3. Histogram representation with frequency distribution of different ELISA-BKM16 results with a panel of 372 samples.

4.3.2 Results of CF performance compared to WB in movement control surveillance

Table 4 shows sensitivity and specificity results comparing CF and the reference test used, WB, for different prevalence levels. Compared to WB, CF sensitivity was 0 (95% CI: 0-0.26), since none of the 12 positive results identified by WB were identified as positive by CF. On the other hand, from the 365 sera identified as negative by WB, 362 were identified as such by CF, with a specificity of 0.99 (95% CI: 0.99-1). None of the 3 positives detected by CF were confirmed by WB.

TABLE 4. Results of sensitivity (Se) and specificity (Sp) calculations, comparing CF to WB as reference. N= 377.

Variables	Estimaded	95% CI
Se	0	0-0.26
Sp	0.99	0.98-1

4.3.3 Results of ELISA-BKM16 performance in movement control surveillance

As shown in Table 5, for the different ELISA cut-off points tested (between 0 and 45), the sensitivity remains at 1 until the cut-off point of 20. From a cut-off point of 0 to 25, specificity increases and, from that point, it remains unchanged in these studies. Thus, at the cut-off point of 20 sensitivity remains at 1, with a high specificity of 0.98.

TABLE 5. Results of sensitivity (Se) and specificity (Sp) calculations, comparing ELISA (10 different cut-offs) to WB as reference. N = 377.

Cut-off	Se	95% CI	Sp	95% CI
0	1	0.74-1	0.64	0.59-0.69
5	1	0.74-1	0.85	0.81-0.88
10	1	0.74-1	0.91	0.87-0.93
15	1	0.74-1	0.95	0.92-0.97
20	**1**	**0.74-1**	**0.98**	**0.96-0.99**
25	0.92	0.62-1	0.99	0.97-1
30	0.92	0.62-1	0.99	0.98-1
35	0.75	0.43-0.95	0.99	0.98-1
40	0.67	0.35-0.9	0.99	0.98-1
45	0.5	0.21-0.79	0.99	0.98-1

4.3.3.1 Receiver operating characteristic (ROC) curve

The results provided in the previous section, which recommended using a cut-off point of 20 in this test based on sensitivity, are also confirmed by the ROC curve presented in Figure 4. This curve shows that the best balance between maximum sensitivity and optimum specificity would be obtained at a cut-off point of 20, thus obtaining the greatest area under the curve.

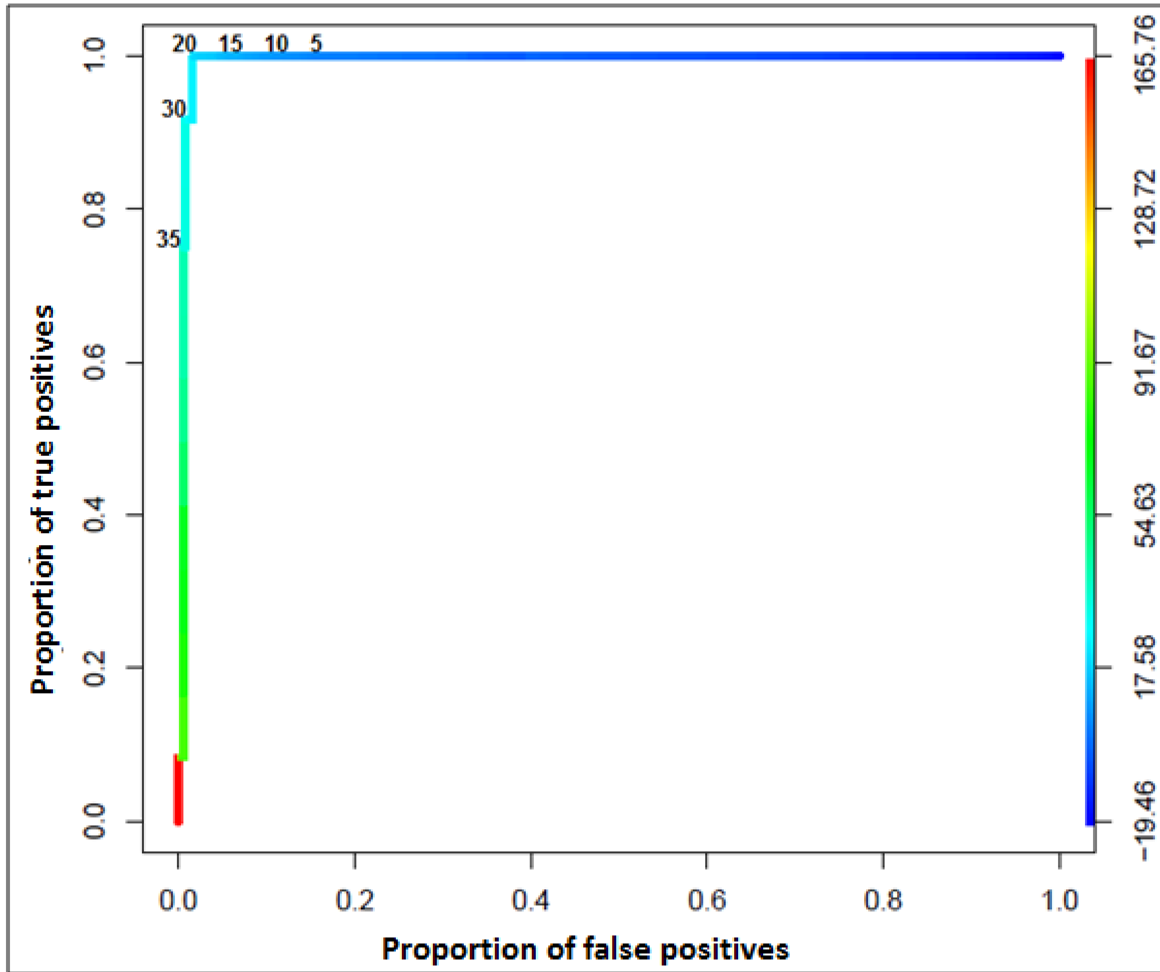

FIGURE 4. Receiver operating characteristic (ROC) curve for ELISA-BKM16 with a panel of 372 samples, evaluated with 10 different cut-offs from 0 to 45.

5. DISCUSSION

This work provides relevant information on the performance of the recently created ELISA-BKM16 compared to CF and WB, in three different assays: one with a known panel, another with samples obtained from surveillance during follow-up, and another with longitudinal surveillance in a controlled environment.

The ELISA-BKM16 developed, as presented in this work, offers good performance characteristics. This ELISA can be used for anti-*B.mallei* antibody detection in equine sera and shows adequate repeatability and reproducibility in development tests (data not shown in this study), which was confirmed by the results of biweekly analyses of positive and negative equines isolated in the described longitudinal study of Cananéia. Besides, it allows for the adjustment of the cut-off point to optimize sensitivity or specificity based on the desired strategy.

In the work presented herein, 100% sensitivity and specificity is achieved in the research made with the known panel. Likewise, the longitudinal study of the animals isolated in Cananéia shows good agreement between the two tests (ELISA-BKM16 and WB). Similarly, in samples of movement control,

ELISA-BKM16 shows very good agreement compared to WB, and may achieve a sensitivity of 100% while maintaining high specificity (98%), as stated in previous studies using the TssB protein (Malik, 2016; Singha et al., 2014). In fact, this good agreement falls within the expected range, since both tests were developed with the same recombinant antigen. The added value of this test is that ELISA-BKM16 performance is at the same level of WB, which is considered a more robust test.

In contrast, the results of comparing CF and WB in movement control samples yield poor agreement, with low sensitivity of CF. This is also observed in the longitudinal study of the animals isolated in Cananéia; even in the euthanized animal with glanders confirmed at necropsy and by PCR, the results of CF were negative in the months prior to death. This indicates that this mare was positive for CF only in the acute stage of the disease, in the first collection of the series conducted by the official laboratory. False negative results can be obtained with CF in chronically infected equines due to the presence of low antibody titers (at levels below the detection threshold of the test), or problems related with complement fixation test supplies (antigen, complement, sheep red blood cells, hemolysin, etc.). Also, low specificity (with false positives) can be observed due to cross-reactivity with other Burkholderia bacteria, or in relation to fragilized (old) red blood cells or low specificity and not inactivated serum (depending on the antigen adsorbed). Souza(2012) also found the same inconsistencies with CF.

Regarding the use in glanders surveillance and control, the results of this work suggest that the ELISA BKM16 is indicated for confirmation of glanders cases both in the acute and chronic stage of the disease. The only inconsistency presented by the ELISA BKM16 compared to WB in the longitudinal follow-up in Cananéia was in an intact male where WB results were consistently positive, while the ELISA showed fluctuating results. It is thought that certain hormone level may interfere with the ELISA.

According to the results of this study, ELISA BKM16 emerges as a potential alternative to CF for glanders surveillance and control, providing good operating characteristics (sensitivity and specificity) in addition to cost-effective advantages due to its easy implementation at the laboratory.

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