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Original Article

Glanders re-emerging in few horses in East-Azerbaijan, Iran

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Summary
Glanders is a zoonosis caused by *Burkholderia mallei (B. mallei).* Glanders has been re-emerging in recent years due to war in the Middle East, unauthorized transfer, the lack of formulated action plans, etc. The prevalence of glanders in Iran and the risk of B. mallei transmission and infection is high, therefore requires the quick identify this disease in animals, particularly in horses. This study investigated glanders re-emerging in horses in East Azerbaijan Province, Iran. From 22 September 2020 to 20 March 2021, six-month periodic tests such as the complement fixation test (CFT) were implemented by the Veterinary Administration of East Azerbaijan to detect glanders in horses. In the case of positive CFT results, the mallein test was conducted. According to the test results, blood samples were taken to culture and prepare serum for the ELISA test. Deep swab samples were collected from nasal mucosa, lymph fluid, and blood. The CFT results indicated 12 horses were susceptible to glanders, and three horses were diagnosed with glanders based on the mallein confirmatory test results. *B. mallei* were not isolated in culturing the samples. Three cases were positive in the ELISA test which was consistent with the CFT and mallein test results. However, the molecular test results were negative. It is challenging to isolate *B. mallei* in the early stage of disease, and the negative molecular diagnostic test result may be misleading in glanders diagnosis. In susceptible cases with a positive CF test result, glanders can be diagnosed by skin mallein and ELISA tests.
Keywords: *Burkholderia mallei,* Glanders, *equine,* Complement fixation test, Mallein test, ELISA.

Introduction

Glanders is one of the most dangerous infectious diseases among equines, especially horses. Glanders is usually chronic in horses and often acute and fatal in mules and donkeys. Glanders is caused by the immobile non-spore-forming gram-negative bacterium *Burkholderia mallei (B. mallei)* (Smith, 2018; Lopez et al., 2003; Whitlock et al., 2007*).*

Equines are the only host of *B. mallei*, and carnivores are infected by eating glanders-infected meat. Glanders is transmitted to animals by discharging secretions from the respiratory tract of the infected livestock (reactor) to common food

and water and through close contact with skin lesions of infected animals or aerosol, mostly in large herds *(*Van Zandt et al., 2013*). B. mallei* are mainly transmitted to humans through occupational exposure, such as frequent and direct contact with infected animals or tools and causes disease in humans *(*Estes et al., 2010; Gilad et al., 2007).

The incubation period lasts from a few days to several months, depending on the infection path and strain severity. Clinical re-emerging and even the death of the horse may occur several months or years after a long incubation period, especially after any stress causing a temperature rise such as vaccination, infectious diseases, poor nutrition, overwork, horseback riding, and displacement (Khaki et al., 2012; Vallat and Caporale, 2012). In general, asymptomatic acute glanders in horses is the main reason for re-emerging the infection in non-native and eradicated regions (WHO, 2018).

Glanders has been eradicated and its incidence has been minimized in most countries in the past century by implementing formulated action plans such as improved diagnosis, trade restrictions with affected countries, the industrial revolution, and not using horses for transportation (Khaki et al., 2012). The re-emerging of war and unrest, followed by widespread poverty in most Middle East countries such as Iraq and Syria, provided the ground for the re-emergence of the disease in these countries (Ellis, 2020). The reports on glanders have increased for the past 15 years in Bahrain (Wernery et al., 2011), Kuwait (Laroucau et al., 2021), the United Arab Emirates (Wernery et al., 2019), Iran (Kianfar et al., 2019), Iraq (Hussein, 2018), Pakistan, and Turkey (Sial et al., 2020).

Glanders is endemic in most world regions, and Iran is still among the main endemic outbreak sites of the disease in West Asia (Khaki et al., 2012). The epidemic occurrence of glanders in 2010 among lions and tigers in Tehran Zoo is one of the new reports of glanders, leading to the death and extermination of several collars (Mardani and Kamali, 2011).

According to World Organization for Animal Health (OIE) and the Centers for Disease Control and Prevention (CDC), *B. mallei* is among the top-ranked organisms in the international community due to transmission through the respiratory tract, the lack of effective vaccines, and intrinsic resistance to antibiotics. Glanders is considered a reportable disease and a bioterrorism factor *(*Lipstiz et al., 2012; CDC, 2000; Riedel, 2004*).* Unfortunately, there is no vaccine or prevention method for this disease, and the primary method for controlling glanders is to identify and exterminate infected animals and observe complete quarantine conditions (Ellis, 2020).

Glanders is diagnosed by different methods such as culturing fresh nodular secretions, ELISA, mallein test, and CFT (Elschner et al., 2019). Isolating *B. mallei* from clinical samples is the major method for diagnosing glanders. However, time-consuming culturing is rarely used for glanders diagnosis due to the sensitivity of this microorganism to environmental changes and being affected by other microorganisms (antagonism). CFT is the major present diagnostic test suggested by OIE for the international trade of horses. In susceptible cases with a positive CFT result, glanders can be diagnosed by the mallein confirmatory test, ELISA, and culturing (WHO, 2018).

Due to the prevalence of glanders in Iran and the risk of *B. mallei* transmission and infection is high, therefore required the quickly identifying this disease in animals, particularly in horses. This study investigated glanders re-emerging in horses in East Azerbaijan Province, Iran.

Materials and methods

Samples

Serum samples were collected from 12 horses susceptible to glanders in clubs by Veterinary Administration experts based on the Glanders Control and Prevention Plan from 22 September 2020 to 20 March 2021 in Tabriz, East Azerbaijan.

Diagnostic tests

Complement fixation test (CFT)

Of 270 serum samples were tested by CFT. Horse serum charged with *B. mallei (*RTCC 2375*)* and the standard negative serum in the laboratory were used for the positive and negative control, respectively. Typically, 1:100 and 1:10 dilutions of hemolysin and complement *(*RVSRI) were prepared, respectively. After titration, the optimal dilutions were prepared for the CF test. The hemolytic system was prepared using veronal buffer *(*1.256 mM $CaCl_2.2H_2O$, 4.132 mM $MgCl_2.2H_2O$, 727.2 mM NaCl, 15.85 mM $C_8H_{12}N_2O_3$, and 9.07 mM $C_8H_{11}N_2O_3Na$), 2% fresh defibrinated sheep blood, and proper titration of rabbit hemolysin. The positive and negative controls were incubated for 30 min at 56°C and then diluted five times with veronal buffer to prepare the test serum samples. In wells of rows B, C, D, and E of a 96-well U-shaped plate, 25 µL of veronal buffer were poured, and 50 µL of diluted serums (diluted by 1:5 in veronal buffer) was added to row A (in wells 1 to 6). Then, 25 µL of diluted serum samples in row A (1:10) was taken and added to row B. The same procedure was performed for rows C, D, and E, and the last 25 µL was discarded. Then, 25 µL of the proper antigen concentration (BioVeta, Czech Republic) was added to all wells in rows A to E, and 25 µL of the titrated complement was added to wells in rows A to E, and the plate was incubated at 37°C for 90 min. Finally, 50 µL of the hemolytic system was added to all wells, and the plate was placed at 37°C for 30 min. Eventually, the plate was centrifuged at 360 g for 3 min and precisely observed by an operator under sufficient light (Elschner et al., 2019).

Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of the test were calculated as follows:

Sensitivity = [True positives / (True positives + False negative)] × 100

Specificity = [True negative / (True negative + False positive)] × 100

Positive predictive value (PPV) = [True positives / (true positives + False positive)] × 100

Negative predictive value (NPV) = [True negative / (True negative + False negative)] × 100

Mallein test

Mallein, a glycoprotein extracted from cultures of *B. mallei*, is used as an antigen in the intrapalpebral, subcutaneous, or ophthalmic test. In this test, 0.1 ml of concentrated mallein PPD is injected intradermally into the lower eyelid and the test is read at 48 h. A positive reaction is characterized by marked oedematous swelling of the eyelid, and there may be a purulent discharge from the inner canthus or conjunctiva. This is usually accompanied by a rise in temperature. With a negative response, there is usually no reaction or only a little swelling of the lower lid (WHO, 2018).

ELISA

Equine serum samples were assayed by the ELISA. The pool of positive and negative serum samples was included on each ELISA plate along with the field serum samples to monitor the accuracy of the assay. Briefly, 90 µl dilution buffer and 10 µl of the serum samples were added to an antigen-coated well. After 45 min incubation at 25°C, wells were emptied, washed three times and incubated with the conjugate for 30 min at 25°C. Washing was repeated three times. Then 100 µl of conjugate (1x) was added and after incubation for 30 min the 3-fold washing procedure was repeated. Finally, 100 µl of the substrate solution was added and after 15 min incubation at 25°C the reaction was stopped by 100 µl of the stop solution and OD 450 nm value was recorded (Singha et al., 2014; Elschner et al., 2019).

Culture

Deep swabs of the bilateral nasal mucosa and lymph fluid and blood were prepared. After transferring to the Tryptic Soy Broth (TSB) medium (Merck, Germany) containing 4% glycerol, the collected samples were sent to the Reference Laboratory of Razi vaccine and serum research institute (RVSRI) to be cultured. Nasal nostril swabs, lymph, and blood were cultured in the biphasic nutrient broth and the glycerinated nutrient agar medium containing 4% glycerin with polymyxin B (merck, Germany), bacitracin, and

actidine antibiotic (to turn the medium selective) (WHO, 2018). After 48 h of incubation at 37˚C under aerobic conditions, slides were prepared from colonies grown on the solid medium and then examined by gram staining.

Extraction of genomic DNA

To extract the bacterial genome, a loop of the bacterial mass grown on the glycerol nutrient culture medium was taken in biosafety cabinet class II and transferred to 400 µL TE 1X buffer in the microcentrifuge tube equipped with an anti-leakage safety gasket. The bacterial suspension was placed for 20 min in a boiling water bath to deactivate the bacteria. The microcentrifuge tube was then removed from the water bath and centrifuged at 10000 g for 10 min after cooling. The supernatant was taken and filtered by a 0.2-µm filter to ensure the lack of live bacteria in the liquid. Of the filtered suspension, 10 µL was cultured on a blood agar plate followed by incubation at 37˚C for 24 h, and then examined in terms of any sign of probable bacterial growth. After ensuring the deactivation of bacteria, suspensions containing the extracted bacterial genome were stored in a refrigerator or freezer until being used in the molecular assays (Merwyn et al., 2010).

PCR

A specific primer pair of *B. mallei* was used in the PCR assay. A final volume of 12 µL was set in the PCR reaction including 6 µL of the master mix, 1 µL of the solution (10 µM) of each primer pair including 5' GCC CTT GTC GAA TGG CAG T 3' as the forward primer and 5' AAG GCT ATC GAC CGC GAT G 3' as the revers primer, 1.5 µL of the bacterial genome-containing suspension, and 2.5 µL double distilled water. Double distilled water and *B. mallei* genome *(*RTCC 2375*)* were respectively used as the negative and positive controls. The PCR was performed under the following conditions*:* 5 min initial denaturation at 94˚C followed by 34 cycles including 1 min denaturation at 94˚C, 1 min denaturation at 68˚C (to attach primers to the target site on the bacterial genome), 1 min denaturation at 72˚C (for amplification), and eventually 10 min heating at 72˚C (for completing the amplification process). Electrophoresis was performed using Red Safe pre-stained 1% MP agarose with a genetic marker size of 100 base pairs for 90 min at 2 V/cm.

Results

Examination of suspicious cases showed two asymptomatic cases and a case with symptoms such as faintness, anorexia, lack of subcutaneous nodules, severely swollen lymph nodes, and testicular swelling (figure 1). No chronic respiratory disease was found in the history of suspicious cases.

CFT

A total of 12 horses susceptible to glanders were diagnosed by the CF test. Of 270 serum samples tested by CF, 12 (4.45%) serum samples were positive. Complete lysis of sheep blood in the serum, antigen, and complement control wells confirmed the accuracy of the CF test. Moreover, agglutination in the hemolytic system control well shows the accuracy of the CF test (figure 2).

A false positive case and a false negative case were reported in the CF test. The sensitivity and specificity of 91.66 and 99.09% were estimated for the CF test. PPV and NPV were respectively estimated at 91.66 and 99.09%.

The results of the CF test were analyzed by the ELISA kit setup in RVSRI.

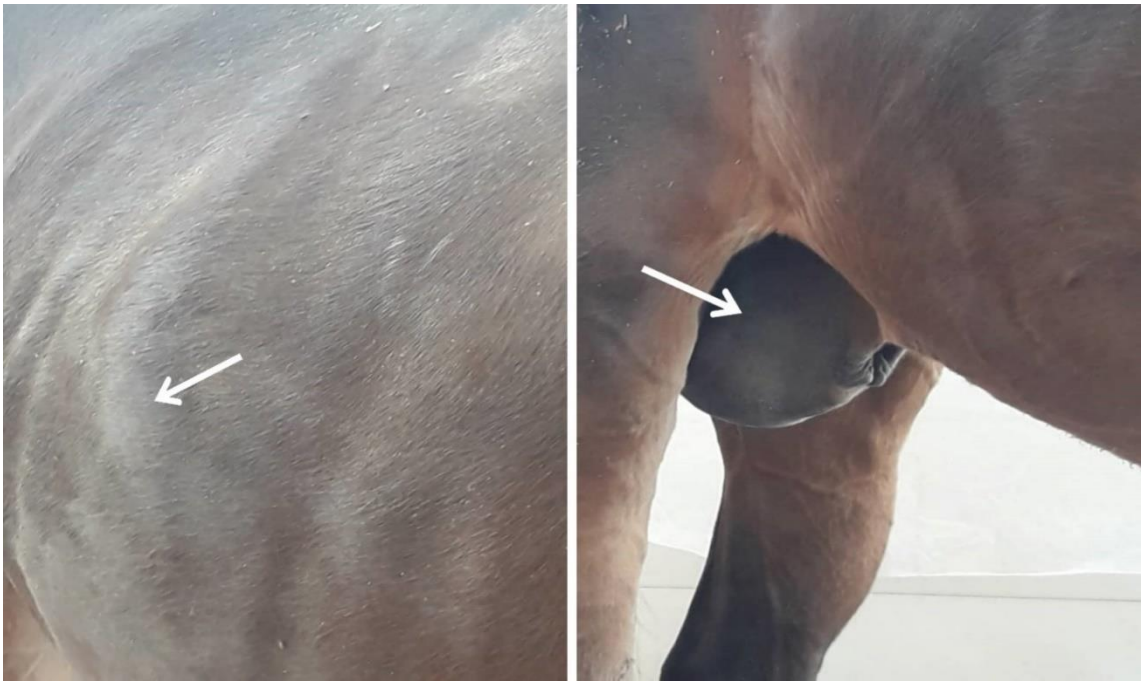


Fig. 1. Severe swelling of the scrotum and swollen lymph nodes.

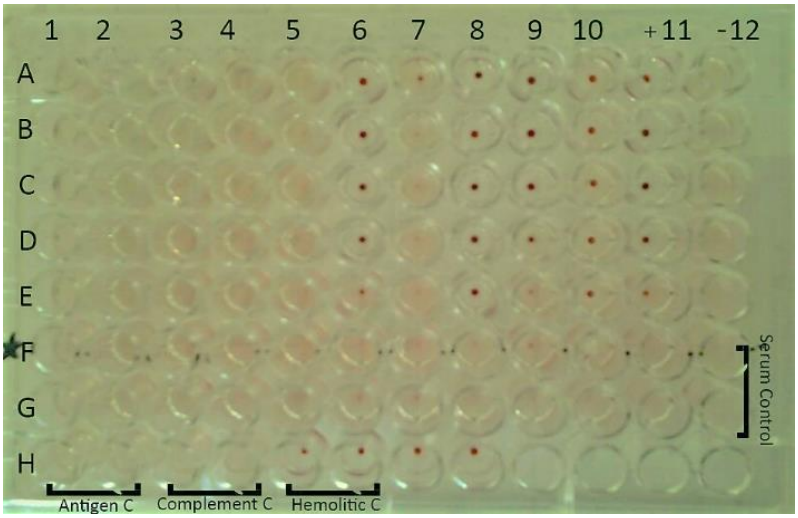
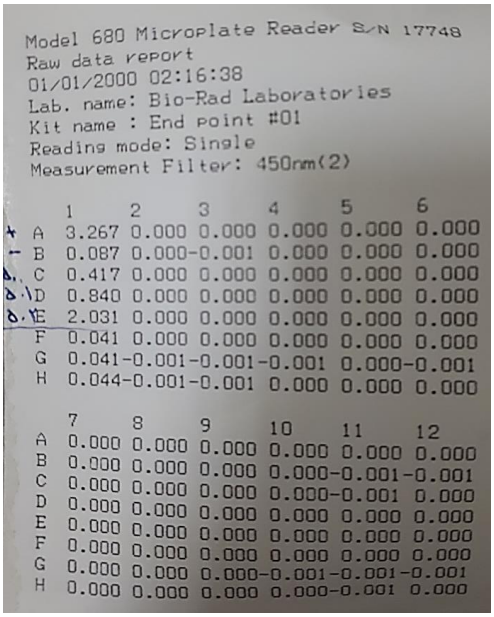


Fig. 2. CF test for equine serums [11: Positive control (agglutination), 12: Negative control (complete blood lysis), Rows F and G: Control serums, Row H (1 and 2): Antigen control, Row H (3 and 4): Complement control, Row H (5 and 6): Hemolytic control]



Model 680 Microplate Reader S/N 17748
Raw data report
01/01/2000 02:16:38
Lab. name: Bio-Rad Laboratories
Kit name : End point #01
Reading mode: Single
Measurement Filter: 450nm(2)

	1	2	3	4	5	6
A	3.267	0.000	0.000	0.000	0.000	0.000
B	0.087	0.000	-0.001	0.000	0.000	0.000
C	0.417	0.000	0.000	0.000	0.000	0.000
D	0.840	0.000	0.000	0.000	0.000	0.000
E	2.031	0.000	0.000	0.000	0.000	0.000
F	0.041	0.000	0.000	0.000	0.000	0.000
G	0.041	-0.001	-0.001	-0.001	0.000	-0.001
H	0.044	-0.001	-0.001	0.000	0.000	0.000

	7	8	9	10	11	12
A	0.000	0.000	0.000	0.000	0.000	0.000
B	0.000	0.000	0.000	0.000	-0.001	-0.001
C	0.000	0.000	0.000	0.000	-0.001	0.000
D	0.000	0.000	0.000	0.000	0.000	0.000
E	0.000	0.000	0.000	0.000	0.000	0.000
F	0.000	0.000	0.000	0.000	0.000	0.000
G	0.000	0.000	0.000	-0.001	-0.001	-0.001
H	0.000	0.000	0.000	0.000	-0.001	0.000

Fig. 3. ELISA test for equine serums

Mallein and ELISA test

Of 12 suspicious cases in the CF test, three cases were confirmed by the mallein test. All three cases were positive in the ELISA test.

Culture and PCR

No *B. mallei* was isolated from the swab culture taken from nasal nostrils, lymph, and blood samples. *B. mallei* were isolated from none of the samples, and the PCR result was negative. The PCR result may be negative in newly infected horses and those with acute or advanced disease.

Discussion

In line with the growth of modernization and urbanization in Iran in the last half-century, the number and scattering of equines herds have been gradually decreased, and their agricultural, nomination and livestock uses have changed to sports and tourism uses. Unauthorized transport of equines or consumption of their meat as livestock food has been the initial source of entry and dissemination of pathogens in the sensitive population in almost all reports on glanders in Iran (Khaki et al., 2012). It seems that equines used for smuggling in western and northwestern borders of Iran are the major endogenic corridor for disease penetration in equine populations in Iran (Price et al., 2012). Currently, Iran Veterinary Organization (IVO) manages the disease in equines by testing and exterminating infected animals based on the CF and mallein test results. However, identifying and eliminating all sustainable glanders sites seem impossible on a large scale considering the volume of this operation. Accordingly, this program can be improved by using all methods and specialized laboratories capable of executing field operations such as sampling and laboratory procedures such as bacterial culture and serum diagnostics.

It seems necessary to rapidly diagnose glanders due to the sudden increase of cases in the Middle East and its outbreak in Iran, the risk of pathogen transmission from neighboring countries through unauthorized equines, and the vital importance of *B. mallei* to be used as a biologic weapon *(*Green et al., 2019). Furthermore, due to the lack of a proper vaccine for disease prevention, the mortality rate amounts to 50% in the case of late diagnosis even with recommended treatments and to 95% without treatment, highlighting the need for rapid diagnosis to prevent mortality *(*Akbarein et al., 2012*).*

Unfortunately, no study has been conducted on livestock infection by this bacterium in Iran in the past decades. According to the Department of IVO Animal Disease Control, no positive case has been reported from 1971 to 1997, and 5, 25, 13, and 3 positive cases have been reported respectively in 1997, 1998, 1999, and 2000. No positive case was reported from 2002 to 2005 *(*Akbarein et al., 2012*)*. Following the unauthorized transport of horses in west Iran, 3, 9, and 37 horses were diagnosed positive by the mallein test respectively in 2006, 2007, and 2008. Two positive cases in Chahar Mahal and Bakhtiari were confirmed by autopsy in the same year. Of 26 cases reported in 2009, 12, 6, 6, and 1 cases were respectively found in East Azerbaijan, West Azerbaijan, Tehran, and Kurdistan. The number of Iranian horses with glanders increased from 5 cases in 1997 to 50 cases in 2010, indicating the risk of glanders re-emerging (Mardani and Kamali, 2011). The occurrence of several epidemics in recent years, including in Eram Zoo, emphasizes the significance of public health against this re-emerging disease (Mardani and Kamali, 2011).

Taghipour et al. (2010) and Khaki et al. (2012) found glanders in Siberian tigers transferred to Tehran Zoo by culturing and molecular methods (Khaki et al., 2012; Taghipour et al., 2011). Mosavari *et al.* have isolated six *B. mallei* strains from equines and tigers in Iran and exactly fingerprinted them by the Variable number of tandem repeat *(VNTR),* Pulsed-field gel electrophoresis (PFGE), and Restriction enzyme analysis (REA) genome techniques (WHO, 2018). Unlike the results of Khaki et al., which emphasized molecular methods, the current study, focused on serological methods. Because, it is challenging to isolate *B. mallei* in the early stage of disease, and the negative molecular diagnostic test result may be misleading in glanders diagnosis. The molecular diagnostic test results in the current study were negative but the CFT was positive. The CFT results indicated 12 horses were susceptible to glanders. Three horses were diagnosed with glanders based on the mallein and ELISA test results. Diagnosis of glanders with both methods yields the same results, but the ELISA test is much faster than the mallein test for diagnosis of equine glanders. Some methods such as the ELISA cause some problems for veterinary authorities and financial losses to animal owners due to false-positive results. The mallein test requires appropriate laboratory equipment and skilled personnel. To quickly and accurately diagnose the disease, especially in areas where animals cannot be kept, new methods (such as CFT) should be used to identify the disease. In susceptible cases with a positive CF test result, glanders can be diagnosed by skin mallein and indirect ELISA tests.

Currently, some projects are implementing in Iran to evaluate the pathogenicity of isolated strains in RVSRI. Accordingly, OIE recommends newer molecular methods in specialized laboratories to formulate diagnostic protocols for glanders *(*Malik et al., 2015*).*

B. mallei capsules may exist in active infections, hiding the bacterium against the immune system. Therefore, it is more challenging to diagnose the disease source so that a tested horse may have a hidden infection not diagnosed by the CF test. Thus, a significant challenge facing veterinary authorities is to control the international traffic of horses by high sensitivity and specificity tests to diagnose hidden Burkholderia mallei. It is noteworthy that OIE recommends the CF test for the international trade of horses, and other tests such as western blotting can also be used to confirm the CFT results.

Only horses in horseback rider clubs are covered by the mallein screening test to control glanders in equines, without covering barking horses and those in traditional centers. Due to the occurrence of acute glanders in mules, they should be considered a possible source of glanders. Fortunately, no human case has not been reported in Iran, but this is not a reason for insignificance and ignoring glanders. Therefore, a larger population of equines, especially horses, should be monitored in Iran. Moreover, all horse keepers should observe high

biosecurity standards ad rapidly and report suspicious clinical symptoms.

Even a sporadic glanders report in Iran is a warning for the health and supervisory institutions. Given problems with the rapid and precise diagnosis of glanders, this organism was diagnosed with high specificity and sensitivity using specific genes according to OIE diagnostic standards (WHO, 2018).

It can be argued that correct and rapid diagnosis of glanders will reduce possible economic damages caused by sales and transportation restrictions of equines, especially horses while reducing the risk of transmission to other hosts such as humans. Accordingly, it is vital to employ internationally accepted and conventional methods and tests for this purpose. Protecting the biosecurity of species and valuable, expensive races of horses in Iran highlights the need for more precise and intelligent application of control and eradication plans and improving laboratory capabilities for glanders diagnosis. By introducing part of facilities for isolating and diagnosing *B. mallei* in RVSRI, we hope that developing communications with other laboratories and participating in international genetic research such as studying the complete genome of this bacterium pave the ground for diagnosing the pathogen and optimizing glanders diagnostic methods in Iran and other regions in the world.

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

The study protocol was approved by the Ethical Committee of the Razi Vaccine and Serum Research Institute, Agricultural Research Education and Extension Organization (AREEO), Karaj, Iran (IR.RVSRI.REC).

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