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# Isolation, serological and molecular methods in screening of *Burkholderia mallei* in East Azerbaijan province, Iran

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## Abstract

Glanders caused by *Burkholderia mallei* is one of the most dangerous zoonotic diseases in solipeds. Clinical diagnosis of this disease in its early stages in horses, is difficult. This study investigated serological and molecular identification of *B. mallei* in East Azerbaijan province. In the third and fourth quarters of 2020, throughout 2021, and in the first and second quarters of 2022, the complement fixation test (CFT) was performed on 350 horses. The malleination was used to confirm the positive CFT cases. Blood samples were taken for culture and for preparing serums to perform the enzyme-linked immunosorbent assay (ELISA). Deep eye discharge, nostril, cutaneous ulcers and lymph fluid swabs were cultured, and polymerase chain reaction (PCR) was carried out. Eleven horses were CFT-positive. Based on the malleination on the 11 horses, six were affected by glanders, five were not affected (false positive), and one horse was CFT-negative despite exhibiting clinical signs. It was positive by malleination, ELISA and PCR. A total number of seven positive cases of glanders were diagnosed. The *B. mallei* could not be isolated, but the *Burkholderia cepacia* complex was isolated in one case. Except for three cases, the results of the CFT, mallein and ELISA tests were consistent. The amount of confidence interval was 95.00%. It is suggested that ELISA could be used as a complement to CFT in screening and, if positive results are observed in one of the tests, the entire herd must be examined more accurately using the mallein and western blot confirmatory tests.



## Introduction

*Burkholderia mallei* causes glanders as one of the oldest zoonotic diseases.[1-3] Given its features, such as low infectious dose, aerosol transmission, lack of suitable vaccines and intrinsic antibiotic resistance, *B. mallei* is placed on earth list of the most important organisms. As one of the bioterrorism agents, glanders is a reportable disease.[4-6]

Due to the repeated occurrence of war and unrest in many areas of Middle East, the reported cases of glanders in Iran have shown a rising trend during the past 15 years.[6] The complement fixation test (CFT) and malleination are performed in Iran Glanders Control and Eradication Program. Despite the many attempts made by the National Veterinary Organization of the Islamic Republic of Iran to perform the mentioned tests and culling the glanders-affected animals, factors such as the smuggling of live animals and expansion of clubs for breeding horses that lack sufficient knowledge about them, have led to the prevalence of glanders in Iran.[2,7]

The mallein test process requires experienced personnel and takes 48 hr. In addition, the repeated injections of inactivated microorganisms cause the production of antibodies against *B. mallei*, leading to false positive results in serological tests.[8] The culture is the gold

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standard for the diagnosis of glanders. However, this method is time-consuming and dangerous. In addition, there are negative results in the culture for various reasons including secondary contamination in the samples, low concentrations of bacteria in the blood, absence of live bacteria and unsuitable sampling sites for diagnosis that are considered the shortcomings of this method.[8,9]

The world organization for animal health (WOAH) considers the CFT mandatory for world trade activities and screening, and recommends it for regulatory research. This test has a specificity of at least 97.00%. Its positive results are confirmed worldwide using confirmatory tests such as malleination, enzyme-linked immunosorbent assay (ELISA) and western blot.[10] Nevertheless, the implementation protocols in this test such as standardizing the antigens and the hemolytic system (complement-hemolysin) are difficult.[11,12] To diagnose glanders, since 2012 WOAH accepted CFT as first choice followed by ELISA and malleinization based on welfare.[13]

Given the prevalence of glanders in Iran and the possibility and risks of transmission of *B. mallei* between solipeds and humans, the use of serological complement methods for rapid and accurate diagnosis of diseases in animals, particularly horses, is especially important. This study was aimed at serological and molecular identification and isolation of *B. mallei* in East Azerbaijan province, Iran.

## Materials and Methods

**Samples.** In the third and fourth quarters of 2020, throughout 2021 and in the first and second quarters of 2022, in the implementation of the program to control and eradicate glanders, CFT was performed once every three months in screening for this disease on 350 horses in East Azerbaijan province, Tabriz. Where the results were positive in the CFT, the mallein test was performed. The approved research project code at Razi Vaccine and Serum Research Institute (RVSRI) was 34-18-1885-017-000176.

**Culture.** Deep swab samples from eye discharge, nostrils, lymph fluid and blood were transferred to tryptic soy broth (TSB; Merck, Darmstadt, Germany) containing 4.00% glycerol (Merck) and taken to RVSRI to be cultured. The deep swab samples were cultured in biphasic nutrient broth and nutrient agar (Merck) containing 4.00% glycerin and the antibiotics polymyxin B, bacitracin and actidine (Merck).[13]

**Complement fixation test.** The CFT was performed with the standard method as described by WOAH.[10] Serum hemolysis (1:5 dilution) was considered negative. The controls of negative, positive, complement/anti-complement and hemolytic systems were remarked. According to WOAH a sample at 1:5 dilution, produced 100% hemolysis = negative; 25.00 - 75.00% hemolysis = suspicious; no hemolysis = positive.

**Enzyme-linked immunosorbent assay.** Indirect ELISA was launched in 96-well microplates (Corning, Kim Seng Promenade, Singapore) coated with 10.00 µg $mL^{-1}$ *B. mallei* (Razi Type Culture Collection RTCC: 2375) lipo-polysaccharide antigen in 0.05 µL carbonate-bicarbonate buffer in pH 9.60 at 4.00 – 8.00 ˚C overnight. The plates were twice washed (Hydroflex; ViraTeb, Tehran, Iran) with phosphate buffered saline with Tween 20 (PBS-T, pH 7.20; Merck, Darmstadt, Germany) and blocked with 1.00% bovine serum albuminin (Sigma-Aldrich, St. Luis, USA) in PBS-T for 2 hr at 37.00 ˚C. Test sera diluted 1:200 in blocking buffer was appended in duplicate wells for 1 hr at37.00 ˚C. Then, the wells were washed five times with PBS-T. Rabbit anti-horse Immunoglobulin G (Sigma, Munich, Germany) antibodies conjugated to horseradish peroxidase diluted 1:10,000 were added to the wells and incubated for 1 hr at 37.00 ˚C. After washing five times with PBS-T, the plate was developed in the dark for 10 min with 100 µL of 3,3',5,5'-tetramethylbenzidine chromogenic substrate solution (Sigma-Aldrich). The reaction was stopped by the addition of 2.50µLHCl and absorbance was measured at450 nm in an ELISA reader (BioTek 800 TS; Agilent, Santa Clara, USA). Finally, the cutoff value for indirect ELISA (iELISA) was determined. Calculations including 95.00% confidence interval was based on standard formulas.

**Mallein test.** In all horses with positive reactions in CFT and iELISA, the mallein test was performed by injecting 0.10 mallein intradermally into the lower eyelid.

**Molecular identification.** To extract the bacterial genome, a loop of the TSB medium was transferred to 400 µL Tris-EDTA (TE) (Sigma-Aldrich) 1.00X 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. The microcentrifuge tube was centrifuged at 10,000 *g* for 10 min and the supernatant was filtered by a 0.20-µm filter to ensure the lack of live bacteria. A final volume of 12.00 µL was set in the polymerase chain reaction (PCR) reaction including 6.00 µL of the master mix, 1.00 µL of the solution (5.00 pmol $\mu L^{-1}$) of each primer pair including Bma-IS407-flip-f (*5'-TCA-GGT-TTG-TAT-GTC-GCT-CGG-3'*) and Bma-IS407-flip-r (*5'-CTA-GGT-GAA-GCT-CTG-CGC-GAG-3'*)based on OIE 2018 recommendation, 1.50 µL (100 to 150 ng $\mu L^{-1}$) of the bacterial genome-containing suspension and 2.50 µL double distilled water ($ddH_2O$). The *B. mallei* and $ddH_2O$were respectively used as the positive and negative controls. The amplification was carried out with conditions includes 5 min initial heating at 94.00 ˚C followed by 34 cycles including 1 min heating at 94.00 ˚C, 1 min heating at 68.00 ˚C, 1 min heating at 72.00 ˚C and eventually 10 min heating at 72.00 ˚C. Electrophoresis was performed using Red Safe pre-stained 1.00% multi-purpose agarose (Roche, Munich, Germany) with a marker size of 100 base pairs (bp) for 90 min at 2.00 V $cm^{-1}$.[13,14]

## Results

The CFT was positive in 11 of the 350 tested serum samples. Complete lysis of sheep red blood cells in the serum control, antigen control and complement control wells indicated the accuracy of the CFT results. In contrast, agglutination in the hemolytic system control well confirmed the accuracy of the CFT results (Fig. 1).

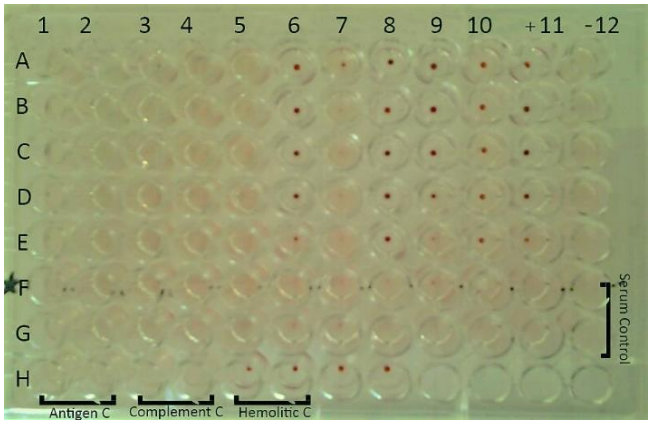


**Fig. 1.** Complement fixation test for equine serums. 11: Positive control (agglutination), 12: Negative control (complete blood lysis), Rows F and G: Control serums, Row H (1 and 2): Antigen control, Row H (3 and 4): Complement control, Row H (5 and 6): Hemolytic control.

Based on the results of the mallein test, six horses (1.70%) were affected by glanders, and five horses (1.42%) were negative (false positive). One horse (0.28%) exhibited clinical signs but was negative in the CFT (false negative). However, it was positive in the malleination, ELISA and PCR tests. *Burkholderia mallei* was not isolated from the cultured samples, however, *Burkholderia cepacia* complex was isolated from the cultured samples in one case. Except for three glanders cases (42.85%), the results of the CFT and those of the malleination and ELISA tests (42.71%) were consistent. Examining the seven horses affected by glanders revealed that three (42.85%) lacked disease symptoms, and four (57.14%) exhibited clinical symptoms, including fever, inappetence, dyspnea, skin and nasal discharge, severely swollen lymph nodes, and hydrocele (Fig 2A-F). Respiratory chronic disease was not found in the history of the cases were CFT-positive and negative by malleination.

Electrophoresis of the PCR products from the initial culture media showed bands in the 989 bp (encompassing the IS07-flip gene) compared to the *B. mallei* positive control. Out of the seven glanders cases (six positive CFT cases were confirmed by malleination .One false negative CFT case was positive by malleination despite exhibiting clinical signs, ELISA and PCR tests) five cases (71.42%) were positive regarding the presence of the primer sequence specific to the IS407-flip genes (Fig. 3). In two cases, PCR yielded negative results.


**Fig. 2.** Clinical symptoms in the horses. **A)** Pustules on the hind legs and suspicious honey-yellow discharge (false negative); **B)** Hydrocele; **C)** Swollen lymph nodes; **D)** Weakness and lethargy; **E)** Severe respiratory symptoms; and **F)** Nasal discharge together with epistaxis.

Since *B. cepacia* was isolated from the horse in selective and differential media in one case, the CVMP23-1/CVP23-2 primers of the SrRNA23 gene specific to this species were used in the second stage to ensure that the isolated samples were belonged to the genus *Burkholderia*. The bands produced by the PCR products were in the 526 bp (Fig. 4A).

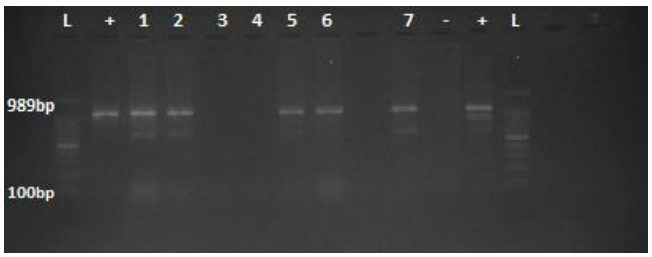


**Fig. 3.** The polymerase chain reaction products electrophoresis from the initial culture (encompassing the IS07-flip gene); Lane L: 100 bp ladder, Lanes 1-7: isolates, Lane -: Negative control, and Lane +: Razi 325 isolate.

Then, simultaneous electrophoresis of PCR products (top row lanes) did not produce 1,051 bp (species-specific 23S rRNA) compared to the *B. mallei* positive control. The PCR products (bottom row lanes) produced 117 bp with the breakpoint cluster region primer (the species-specific *recA* gene) compared to the *B. cepacia* positive control which proved the presence of the *B. cepacia* complex (Fig. 4B).

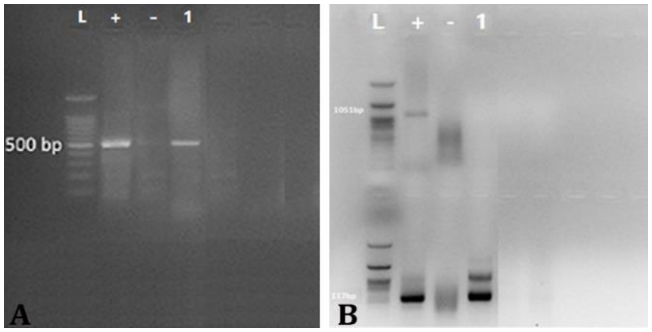


**Fig. 4. A)** Amplification of *Burkholderia* specific 23S rDNA genes (CVMP23-1/CVP23-2 primers); Lane L: 100 bp ladder; Lane +: Razi 325 isolate; Lane 1: isolate; Lane -: negative control; **B)** Amplification of 23s rDNA and *recA* genes. Top row: Amplification of the 1,051 bp fragment related to the 23s rDNA gene specific to both species of *B. mallei* and *B. pseudomallei* (primers mp23-2, and vmp23-1) and bottom row: The amplification of the 117 bp fragment related to the *recA* gene specific to the species *B. cepacia*. Lane L: 100 bp ladder; Lane +: isolated DNA of *B. pseudomallei* (Top row) and *B. cepacia* (lower row); Lane -: negative control; Lane 1: isolate.

The *B. cepacia* was isolated only from sample 7 among the cultured swab samples from eye discharge, nostrils, cutaneous ulcers, lymph fluid and blood. *B. mallei* was isolated from none of these culture media. Five cases of the CFT results were confirmed by the ELISA kit set up at RVSRI. As shown in Table 1, the CFT, malleination, ELISA, and PCR results were consistent in four cases (71.42%) namely samples 2, 3, 4 and 5. Despite clinical signs in sample 1, the CFT result was negative, however, the ELISA, malleination and PCR results were positive. Except for samples 1, 6, and 7, all CFT results were consistent with PCR results. According to the results, seven horses (2.00%) were confirmed by the malleination test to be affected by glanders and thus were euthanized.

**Table 1.** Results of complement fixation test (CFT), malleination, enzyme-linked immunosorbent assay (ELISA), polymerase chain reaction (PCR) and culture in confirmed samples

| Methods | Tests | Samples | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| Indirect | CFT | - | + | + | + | + | + | + |
| | Mallein | + | + | + | + | + | + | + |
| | ELISA | + | + | + | + | + | + | - |
| Direct | Culture | - | - | - | - | - | - | + |
| | PCR | + | + | + | + | + | - | +(*recA*) |

## Discussion

Iran is known as one of the glanders foci in the Middle East and the presence of three strains with very high heterogenicity indicated the entry of *B. mallei* strains, as an emerging pathogen, to Iran from the borders. Consequently, among the major dilemmas in controlling and eradicating this disease in Iran are the illegal traffic of infected odd-toed ungulates in border areas, insufficient funding for implementation costs, and the lack of suitable laboratory facilities leading to the failure in suitable and early diagnosis of the causative infectious agent of glanders especially for the chronic cases of this disease. Accordingly, given the sudden rise of this disease in the Middle East and its prevalence in Iran, identifying and isolating healthy carriers and infected animals can be helpful to the National Control and Eradication Program of Glanders.[15-17]

Previous studies have shown that CFT is somewhat non-specific. It yields false positive results because it interferes with the malleination test and because of the possibility of cross-reactivity between *B. mallei* and *Burkholderia pseudomallei*. It also yields false negative results due to the intracellular nature of the pathogen and humoral immune deficiencies. The literature suggests that clinical and non-clinical carriers cause false negative in testing. False negative results are shown in weak and pregnant animals due to weak immune system, entry of the pathogen into the intracellular cycle (inside the macrophages in the liver, spleen, and lungs), the state of latency in microbial pathogenesis in the early stages of the disease and the impossibility of its detection by the humoral immune system. In addition, the stockman doubts about receiving compensation for his losses and the weakness in the quarantine system inclines the stockman to take the infected livestock out of the stables and sell them, thereby, spreading the disease.[17-19]

Based on the clinical observations of a veterinarian a clinical case suspected of being affected by glanders was reported in this study. The CFT yielded a negative result, however, the malleination, ELISA, and PCR results were positive. Since the malleination, ELISA, and PCR tests were positive, the CFT test was performed in all 40 horses in the club. Seven horses were CFT-positive, of out which, three were also positive by malleination and PCR tests.

Although CFT are recommended by the WOAH however, many routinely used detection tests may present misleading results. Therefore, It is suggested that PCR be used as a complement to CFT in screening, and where positive results are observed in one of the tests, the entire herd must be examined more accurately using the mallein and western blot confirmatory tests. Abreu *et al*. showed that CFT with the United States Department of Agriculture (USDA) and antigens from CCPro GmbH (Oberdorla, Germany) in combination with PCR to increase sensitivity, may be useful for diagnosis of chronic glanders.[12]

Laroucau *et al*. recommended the PCR systems be used for the molecular diagnosis of glanders, especially in regions where the circulating *B. mallei* strains have not yet been fully genetically characterized.[20]

Consequently, the false negative cases could be very important as sources of infection and cause an epidemic in the herd. The species in the *B. cepacia* complex isolated in this research can survive in soil and moist environments for a long time, infect horses, and interfere with the glanders diagnostic test. Therefore, given the importance of this issue, the indirect ELISA test was also designed to identify other pathogenic *Burkholderia* spp.

Mosavari *et al.* examined two CFT-negative horses with suspicious clinical signs in Alborz province, Iran. They noticed that the sensitivity of identifying the infected horses could be raised using several approved tests. After identifying the infected horse, malleination test was performed on the horses in the entire herd. Comparing their results with ours can lead to the conclusion that the likelihood of identifying the cases infected with glanders will increase by performing the diagnostic tests simultaneously.[21] Similar to the present research, Mosavari *et al.* observed clinical signs of glanders in one of the horses clinically examined in Kermanshah province, Iran, in 2018. The CFT results (repeated five times) inthe suspected horse were negative. Immediately after that, blood samples and samples from the nodules located along the pathway of the lymphatic vessels and from the nostrils were taken and cultured. The PCR was performed twice and the results confirmed the presence of *B. mallei* in the cultured samples.[21]

Elschner *et al.* compared the commercially available glanders ELISA (GLANDA-ELISA) test with the CFT and concluded that ELISA was considerably more specific than CFT (99.80 versus 97.00%).[19] Hence, ELISA can be a suitable alternative confirmatory test in world trade. According to Table 1, this study confirmed these results and suggested the simultaneous use of ELISA and CFT.

Shakibamehr *et al.* compared western blot, CFT, and ELISA tests in diagnosing glanders and reported that their sensitivity and specificity were (95.00 and 100%), (100 and 85.00%), and (98.50 and 95.70%), respectively. Western blot and ELISA were considerably more specific than CFT, but ELISA was less sensitive than CFT.[17] According to the results of this study, researchers should adopt parallel testing (using tests with sensitivity and specificity higher than 90.00%) to increase the accuracy of the results if they are similar.

Yazdansetad *et al.* suggested that western blot should be used as a confirmatory test to increase specificity in diagnosing glanders. They also proposed that western blot could be employed as the initial, reliable and diagnostic test in the WOAH guide for eradication programs.[6]

Recent studies comparing ELISA and CFT concluded that the specificity and sensitivity of ELISA exceeded 99.00% using the recombinant antigens Type VI secretion system sheath protein (TssB) and *Burkholderia* intracellular motility A protein (bimA), and ELISA exhibited much higher sensitivity than CFT. In addition, there was no cross-reactivity between ELISA and the recombinant antigen BimA in detecting *B. mallei* via seropositivity to *B. pseudomallei*.[19,22-24] Unlike this research, natural antigens were used in our study; otherwise, ELISA would exhibit higher specificity.

Since *B. mallei* is an intracellular pathogen, the humoral immune system may fail to detect it rapidly. Furthermore, the humoral immune system has lower access to intracellular bacteria, delaying the detection of *B. mallei*.[25] Therefore, negative serological results do not rule out the presence of *B. mallei* (false negative). False-negative results lead to the spread of the disease, considerably harm the health of humans and animals and the economy, and cause security risks.

Accordingly, considering the efficiency of each of the above-mentioned methods, it is suggested that ELISA should be used as the complement test during the screening stage along with CFT. Where the results are positive in one of the mentioned methods, the entire herd must be examined more accurately using the malleination and western blot tests. In addition, where further investigation is needed, sample culture, PCR, and inoculation of the animals should be carried out.

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## Conflict of interest

The authors declare no conflict of interest.

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