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Original Article

Isolation and Purification of the *Burkholderia Mallei* Antigenic Proteins and its Use in Diagnostic Tests

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ABSTRACT

Glanders is a contagious infectious disease caused by *Burkholderia mallei* that affects both solipeds and carnivores. This disease occasionally leads to human infection through direct contact between humans and infected animals. The recent rise in glanders prevalence has intensified focus on control and eradication programs, emphasizing accurate diagnosis of infected cases using high-performance test. To this end, antigenic proteins were purified from *Burkholderia mallei*, and the mallein test was optimized using these purified proteins. Finally, the efficacy of antigenic proteins was evaluated using the complement fixation test (CFT). The laboratory strain of *B. mallei* was selected,and proteins from inactivated bacteria were precipitated using ammonium sulfate (AS) and trichloroacetic acid (TCA). An optimal precipitation method was selected, and the proteins were purified using size exclusion chromatography (SEC) and high-performance liquid chromatography (HPLC). Brute mallein was also prepared for comparison. The protein profile of the samples was analyzed using SDS-PAGE. The mallein test was also performed, and results were evaluated using CFT. The AS method was identified as the optimal precipitation method. The protein profile exhibited a range of proteins from low to high molecular weights, appearing as a smear in the brute mallein. Mallein test using AS-participated proteins, the first SEC fraction and the second HPLC fraction yielded significant results, demonstrating erythema diameters of 18.46, 21.70 and 25.37 mm, respectively. These mallein test results were confirmed by CFT. The findings indicated that the purified antigenic proteins improved both mallein test and CFT results. Consequently, these proteins can diagnose glanders correctly and increase the accuracy of the mallein test and CFT.

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1. Introduction

Glanders is a zoonosis bacterial disease caused by *Burkholderia mallei*. This bacterium transmits dangerous and fatal infections in solipeds, particularly equines, due to the absence of effective and definitive treatments and potential aerosol transmission. The disease may also spread to humans through direct contact with infected animals, resulting in death in most cases. Consequently, the bacterium has potential for use in bioterrorism and biological weapons (1-3).

Cases affected by chronic glanders typically suffer from the disease for several months; while they may initially show signs of improvement, they ultimately either succumb to the disease or continue to remain as a hidden case with only apparent recovery. Infected or apparently healthy carriers are the primary source of infection and should be promptly identified and eradicated (1, 4).

Considering the widespread prevalence of glanders in Iran and the Middle East, and inadequate efforts of certain neighboring countries to control and prevent this disease, the Iran Veterinary Organization allocates significant financial resources for testing and euthanizing solipeds. Nevertheless, glanders persists and occasionally emerges as short epidemics; therefore, Iran remains a global epicenter for this disease (5).

The recent rise in glanders prevalence in the Middle East, emergence of new epidemics, ease of disease transmission, absence of an effective vaccine for preventing glanders, and potential weaponization of the pathogen collectively pose a serious threat to the global health system (6, 7). The Prompt and accurate diagnosis of glanders is of utmost significance. The international community requires access to information about infected cases and their proper diagnosis in Iran, necessitating extensive studies and advanced diagnostic tests to effectively manage the glanders crisis.

Solipeds carrying *B. mallei* are currently detected using the mallein test, which involves purified protein derivative (PPD) or brute mallein (1, 8). Using unpurified proteins in mallein testing causes different delayed hypersensitivity (DHS) levels, necessitating optimization through selection of effective (antigenic) proteins associated with the disease.

Moreover, the mallein test involves injecting a substance into the equine's lower eyelid, any error in the injection process is associated with the risk of injury to the animal's eye. Administrating and interpreting the test results is a highly challenging with significant potential errors task and should be carried out solely by a qualified veterinarian. Therefore, replacing the mallein test with alternatives, such as CFT, is necessary to ensure that all pertinent procedures are carried out within the laboratory setting, allowing for effective control and minimizing potential errors (9-11). Recently, some studies have explored diseaseoccurrence and treatment of based on their antigenic proteins. Such an approach can also be used to evaluate glanders (12, 13).

Purifying *B. mallei* antigenic proteins for PPD production would enhance mallein test efficacy. In addition, the use of antigenic proteins in CFT as a complementary or alternative test can yield more acceptable results, improving confidence in positive case reporting. This study aimed to improve the mallein test results using purified antigenic proteins. Subsequently, the purified PPD efficacy in both the mallein test and CFT is evaluated by analyzing the detection results.

2. Materials and Methods

2.1. Bacterial Strain and Cultural Conditions

The laboratory strain *B. mallei* 325 (RTCC 2375) employed in this study was obtained from the mallein laboratory at Razi Vaccine and Serum Research Institute (RVSRI), Karaj, Iran. *B. mallei* was grown for 30 days at 37°C on Nutrient agar and Dorset-Henley medium containing 4% glycerol. Then the grown bacteria were inactivated at 70°C for 30 minutes (14). Bacteria were cultured on blood agar culture medium to ensure inactivation of the strains.

2.2. Cell Disruption

2.2.1. Sonication

Cell lysis for protein extraction was performed according to the method described recently (15). Briefly, the culture pellet was centrifuged at 9,000 g for 15 minutes at 4°C, then washed thrice with ice-cold phosphate buffered saline (0.01 M PBS, pH 7.0),and was resuspended in PBS containing 1 mM phenylmethane sulfonyl fluoride ,and sonicated on ice at 90% amplitude for 5 cycles of 1 minute pulsing with one- minute rests between the pulses (Hielscher-Ultrasound Technology, PN-66-NNN, Germany), then centrifuged at 9,000 g for 10 minutes. The supernatant was treated with 1 mL lysis buffer (Urea 8 M, Tris-HCl 10 mM, $Na_2HPO_4.7H_2O$ 100 mM, EDTA 1 M, SDS 1%).

2.3. Filtration and Collection of Bacterial Extracts

The sonicated bacteria were passed through a Buchner funnel to strain cell debris , then filtered under pressure using K7 and 0.22 µm filters (Millipore, USA). The collected bacterial extracts were stored at 4°C.

2.4. Protein Precipitation

The sonicated solution was divided into two equal volumes. Proteins from each volume were precipitated using trichloroacetic acid (TCA) and ammonium sulfate (AS), respectively.

2.4.1. TCA precipitation

The sonicated mixture was mixed with 40% TCA at a1:9 ratio and stirred at 4°C for 6 hours at 400 rpm. The mixture was then left overnight at 4°C without stirring. The precipitate was centrifuged at 2500 g for 15 minutes after discarding the supernatant the following day. The resulting precipitate was first washed with 1% TCA ,and then with 10% NaCl, each time followed by centrifugation at 2500 g for 15 min. Finally, the precipitate was dissolved in a solvent buffer (3.8 mM $Na_2HPO_4.7H_2O$, 8.3 mM NaCl) with 6.9±0.1 pH (16).

2.4.2. AS precipitation

AS was gradually added to the sonicated mixture over three hours with stirring to achieve 80% salt saturation at 4°C. After 6 hours of stirring 400 rpm and 12 hours without stirring, the precipitate was centrifuged at 2500 g for 15 minutes after removing the supernatant the following day. The pellet was subsequently dissolved in phosphate buffer (PBS; 6.9±0.1). The protein solution was dialyzed against 10- time distilled water for two days, and concentrated with polyethylene glycol (PEG) 6000 to 10%of the initial volume (16).

2.5. Brute Mallein Preparation

B. mallei Strain 325 was cultured on a Nutrient agar medium with 4% glycerol. After 48 hours, the grown colonies were transferred to a 10 L beaker containing Bouillon medium with 4% glycerol and incubated 30 days at 37°C. The grown bacteria were inactivated at 100°C for 1 hour. The beaker contents were passed through a Buchner funnel to clear cell debris and then filtered using an EKS filter. The filtered liquid was concentrated at 70°C to the one-tenth of the initial volume. Phenol was added to a final concentration of 4% (7).

2.6. Potency Assay

Precipitated proteins and brute mallein were tested for their potency based on six- point assay, by sensitizing guinea pig models according to world organization for animal health (WOAH) recommendations (1, 17).

Nine male outbred guinea pigs weighing 450-550 g, obtained from RVSRI animal care unit were divided into three groups. Throughout the study, the water, food, temperature, and humidity levels of the guinea pigs were monitored daily to ensure their health. A suspension containing inactivated *B. mallei* combined with incomplete Freund's adjuvant was prepared to sensitize the guinea pigs. Guinea pigs received intramuscular injections injections (0.5 mL) of inactivated *B. mallei* suspension (0.01 mg/mL in incomplete Freund's adjuvant) on days 0, 14, and 28. One month after sensitizing the guinea pigs, they were injected with 0.1 mL of brute mallein (Mb group), PPD precipitated with TCA (PPD_T group), and PPD precipitated using AS (PPD_A). The experimental groups received 0.01 mg/mL injections, while the control group received 0.1 mL of PBS; the injections were administered intradermally. The test results were read based on the size of erythema and oedema (mean diameter of two perpendicular lines) at the injection site of the animals after 24 hours. The Diameters above 8 mm were regarded as positive reaction.

2.7. Complement Fixation Test (CFT)

CFT was carried out using the standard method as described by WOAH (1, 18).

Briefly, Hemolysin and complement (RVSRI, Iran) were diluted at 1:100 and 1:10, respectively, and the most suitable dilution for CFT was prepared following titration. Veronal buffer ($CaCl_2.2H_2O$, 1.256 mM; $MgCl_2.2H_2O$, 4.132 mM; NaCl, 727.2 mM; $C_8H_{12}N_2O_3$, 15.85 mM; $C_8H_{11}N_2O_3Na$; 90.7 mM), defibrinated fresh sheep blood (2%), and the proper titer of guinea pig hemolysin were used to prepare the hemolytic system. The samples (including brute mallein, sonicated sample, proteins precipitated with TCA and AS, positive control (*B. mallei* antigens for BRC; Bioveta, Czech Republic) ,and negative controlwere incubated at 56ºC for 30 minutes , then diluted five times using Veronal buffer. A 96-well round-bottom microtiter plate was filled with 25 µL of Veronal buffer, and 50 µL of diluted samples were added one at a time into the wells. After adding 25 µL of the titrated complement to each well, the plate was incubated at 37°C for 90 minutes. After adding 50 µL of the hemolytic system to each well, the plate was centrifuged for 1 minute at 600 g and incubated for 30 minutes at 37ºC.

The results were interpreted as negative when 100% hemolysis was observed, inconclusive with 25–75%

hemolysis, and positive when no hemolysis was detected (at 1:5 dilution).

2.8. Chromatography

The best precipitation method was selected based on the mallein test and CFT results. Resulting proteins underwent purification through two chromatography steps.

2.8.1. Size-exclusion chromatography (SEC)

The protein solution was sterilized using 0.45 µm filter and applied to Sephadex G-50 column (2×150 cm), at 4ºC. The column was equilibrated with 3.8 mM PBS (pH 7.0) and eluted with the same buffer. Fractions of 3 mL were collected at 6 mL/h flow rate 4°C and its absorbances were recorded at 280 nm (19).

2.8.2. High-performance liquid chromatography (HPLC)

SEC fractions (100 µL injection volume) were analyzed on a HPLC System fitted with a reversed-phase analytical column (Waters® XSelect CSH C18, 4.6 × 100 mm, 5 µm) and a C18 SecurityGuard™ cartridge (Phenomenex) in series. The samples were centrifuged at 2800 g to remove the precipitated proteins and the supernatant was applied on HPLC column, C18 (H_2O, 0.1% trifluoroacetic acid), and eluted with a concentration gradient of solvent B (acetonitrile, 0.1% trifluoroacetic acid) from 0 to 100%, at 0.3 ml/minutes flow rate during 50 minutes. The fractions were monitored at 280 nm (20, 21).

Mallein test and CFT were performed for fractions and subfractions obtained from SEC and HPLC, according to the method described above.

2.9. Protein Assay

The protein concentration of the solutions precipitated with TCA, AS, and brute mallein was determined using the Kjeldahl method. Additionally, the protein content of the fractions obtained from SEC and HPLC was also measured by the Lowry method using bovine serum albumin (1 mg/mL) at 280 nm (22, 23).

2.10. Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The molecular weight and protein profile were determined using SDS-PAGE, according to the Laemmli method. A 4% stacking gel and a 15% separating gel were prepared and stained with Coomassie blue and silver nitrate (24).

3. Results

3.1. Bacterial culture and precipitation

After 48 hours, *B. mallei* colonies were observed on the culture medium (Figure 1A). The proteins obtained by the (Figure 1B) and AS (Figure 1C) methods were precipitated after mass cultivation. The proteins precipitated from the AS stage were dialyzed (Figure 1D).

3.2. Chromatography

SEC yielded three peaks corresponding to fractions F_1 to F_3 (Figure 2A), and subsequent purification by HPLC resulted in the isolation of five peaks, namely sub-fractions F_1A to F_1E (Figure 2B). The SEC fraction F_2 and the HPLC sub-fraction F_1A exhibited the highest optical density (OD) and protein concentration.

3.3. Protein assay

The protein concentrations of the samples,including the TCA-precipitated solution, concentrated AS, and brute mallein,were determined using the Kjeldahl method. The values obtained were 4.63, 2.92, and 2.78 mg/mL, respectively. After concentrating, the protein concentration of the F_1, F_2 and F_3 fractions was determined to be 2.10, 4.80 and 0.85 mg/mL, respectively. Moreover, the protein concentrations of the concentrated F_1A to F_1E sub-fractions were 1.06, 0.77, 0.071, 0.003 and 0.016 mg/mL, respectively.

3.4. SDS-PAGE

The protein compositions of all samples were analyzed using SDS-PAGE, and their protein profiles were obtained, as shown in Figure 2. The protein profile of sonicated bacteria and TCA-precipitated proteins exhibited a significant abundance of proteins spanning a wide range of molecular weight proteins from low to high. This profile appeared as a smear through brute mallein. The AS-precipitated proteins revealed the presence of a complex mixture of low to high-molecular-weight proteins, albeit in reduced quantities (Figure 3A). The results showed the presence of proteins with molecular weight of 60 kD or higher, 25-50 kD, and 30 kD or lower in F_1, F_2, and F_3, respectively (Figure 3B). 40, 50, and 66 kD proteins were detected in F_1A and F_1B. In other sub-fractions, the protein concentration was too low to produce a distinct band on SDS-PAGE. A standard protein, with a band around 66kD, was also observed in F_1B (Figure 3C).

Figure 1. Culture of *Burkholderia mallei* (**A**); Protein precipitation by TCA (**B**) and ammonium sulfate (**C**); Dialysis of precipitated proteins with the ammonium sulfate (**D**).

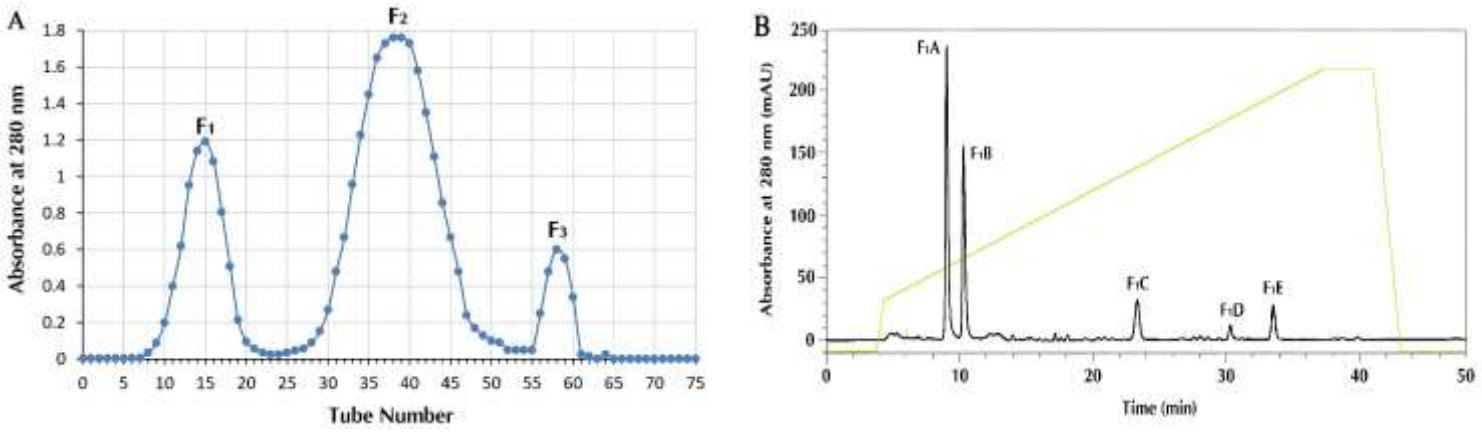

Figure 2. Chromatogram obtained from SEC (**A**) and HPLC (**B**).

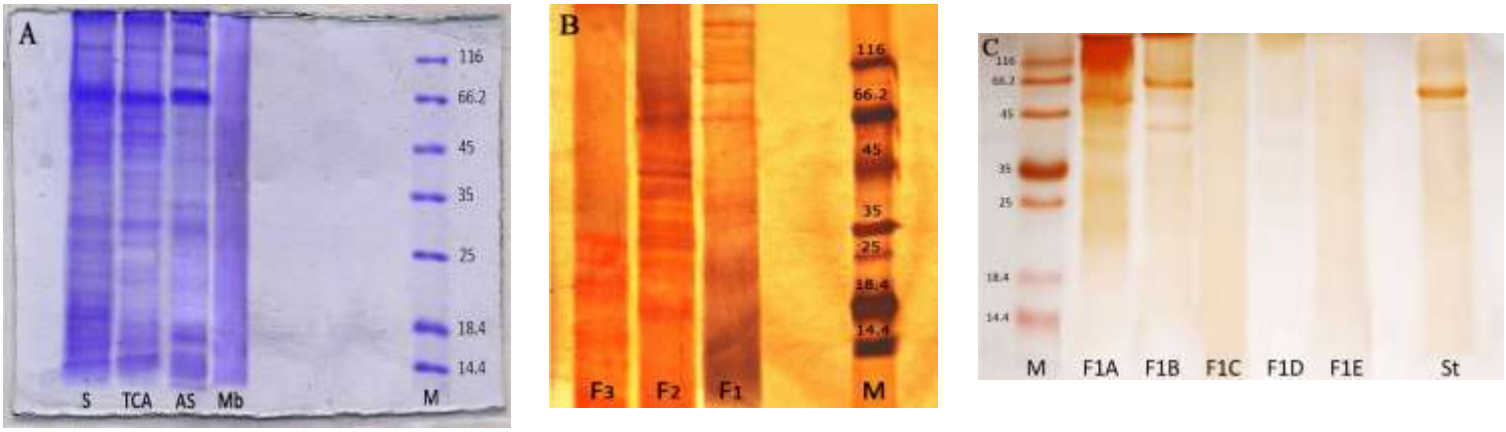

Figure 3. SDS-PAGE profile of precipitated proteins (**A**) and all fractions obtained from SEC (**B**) and HPLC (**C**)
A [M: Protein standard marker; Mb: Brute mallein; AS: Precipitation with ammonium; TCA: Precipitation with trichloroacetic acid; S: Sonicated bacteria]; **B** [M: Protein standard marker; SEC fractions (F_1, F_2, and F_3)]; **C** [M: Protein standard marker; HPLC fractions (F_1A, F_1B, F_1C, F_1D, F_1E,) and St: Standard Antigen].

3.5. Potency assay

After sensitizing 12 guinea pigs and analyzing the erythema caused by injecting brute mallein, (Mb group), TCA-precipitated PPD (PPD_T group), and AS-precipitated PPD (PPD_A) the mallein test in the PPD_A group showed a significant difference compared to the other groups (Figure 4). F_1 and F_1B caused the largest erythema diameters of 21.70 and 25.37 mm in the SEC and HPLC groups, respectively (Table 1).

3.6. CFT

The initial CFT demonstrated that the AS-precipitated protein yielded superior results, as evidenced by complete hemagglutination in well 1 at a 1:1 dilution. The solution subjected to sonication and TCA treatment did not exhibit complete hemoagglutination but showed varying degrees of hemoagglutination and hemolysis across all wells.

The results indicated that F_1 induced complete hemoagglutination up to well 7 (at a 1:128 dilution), followed by the initiation of hemolysis from that well; F_2 exhibited hemoagglutination and hemolysis up to well 3, with complete hemolysis observed from well 3 onward (at a 1:4 dilution); and F_3 induced complete hemolysis at all dilutions (Figure 5A).

During the second step, CFT demonstrated that F_1B performed better than other sub-fractions, as evidenced by complete hemagglutination observed up to well 10 (at a 1:512 dilution). F_1A showed slight hemagglutination in both the first and second wells (at a 1:1 and 1:2 dilutions). Except for F_1B, all other sub-fractions and brute mallein induced complete hemolysis (Figure 5B). The occurrence of complete hemolysis and hemagglutination in the negative and positive control wells confirmed the accuracy of the CFT results (Figure 5 A and B).

4. Discussion

Glanders is a highly infectious and dangerous bacterial disease that persists in solipeds and occasionally affects humans. Iran is inadvertently at risk of glanders due to its proximity to neighboring countries with a high prevalence of the disease, such as Afghanistan, Pakistan, and Iraq. Consequently, Iran is recognized as an endemic center for glanders, alongside other Middle East countries.The potential entry of unauthorized solipeds to Iran underscores the importance of accuratelyidentifying glanders cases (7).

WOAH recommendations emphasize the production of PPD-mallein (1). Currently, in Iran, the mallein test is performed using brute mallein. This protein complex is associated with varying levels of DHS. The variability in the mallein test may be attributed to the inactivation of bacteria at high temperatures or prolonged heating during the protein composition to concentrate the mallein solution. Such heating causes the proteins denaturation, significantly reducing their biological activity. Analyzing the SDS-PAGE profile in this study confirmed that all proteins were denatured and smeared (Figure 3). To more accurately identify infected cases, optimizing this test to minimize its variability and produce a high DHS product is necessary.

Potency assessment results indicated that protein precipitation with AS is a more effective method. When compared under identical conditions, AS demonstrated superior results to TCA. The lower potency of the TCA can be attributed to its denaturing effect on protein structure, which often results in the removal of bacterial carbohydrates and lipids or their binding to proteins.

In addition to proteins, these carbohydrate and lipids are crucial in developing bacterial antigenic properties. Consequently, the reduced efficacy of this method can be attributed to the removal of carbohydrate and lipid antigens,such asglycoproteins and lipoproteins from the PPD-mallein product. In contrast, brute mallein and TCA precipitation yield significant quantity of antigens from *B. mallei*, which may be detected in *B. pseudomallei* and other families, such as Pseudomonas.

Therefore, false positive reactions may be observed in cases where brute mallein is used. The precipitate was separated and examined in precipitation with AS and a concentration gradient at each stage. Additionally, a higher AS proportion was added to the supernatant. Eliminating proteins at each stage reduced the likelihood of false positive reactions compared to cases where brute mallein was used. Hence, the potency of PPD-malein produced using the AS method was significantly increased due to the elimination of commonly found antigens.Verma et al. (1994) investigated the potency of relatively pure proteins for the mallein test to detect glanders, isolating PPD-mallein from *B. mallei* through precipitating bacterial proteins with TCA and AS, followed by purification using gel filtration chromatography. They found that TCA-precipitated proteins of relative purity exhibited the same potency and innocuity as the standard PPD-mallein. In contrast, AS-precipitated proteins elicited a nonspecific reaction. They further observed that the activity of PPD-mallein

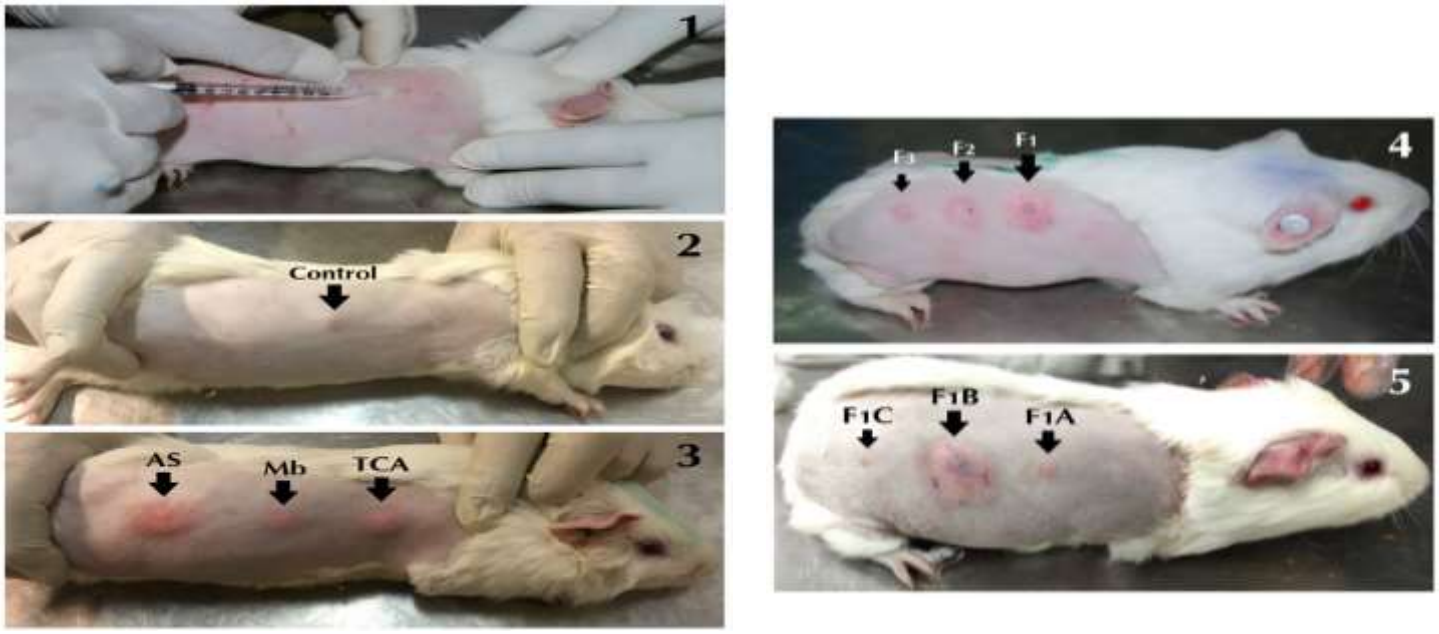

Figure 4. Mallein test [Injection of *Burkholderia mallei* proteins into sensitized guinea pig (**1**); Erythema developed in the control group (**2**); Mb, PPD_T, and PPD_A groups (**3**); SEC group (**4**) and HPLC group (**5**).

Table 1. Mallein test in experimental groups.

	Treatments	Injection[1] (mm)	Injection[2] (mm)	Injection[3] (mm)	Mean±SD
	Control	2.92*	3.16	2.79	2.97±0.15
	Mb	11.24	12.09	12.91	12.08±0.68
	AS	18.71	18.22	18.45	18.46±0.20
	TCA	14.63	13.98	14.01	14.21±0.29
SEC	F_1	22.10	21.08	21.90	21.70±0.44
SEC	F_2	10.47	11.08	10.16	10.57±0.38
SEC	F_3	6.11	5.21	6.45	5.92±0.52
HPLC	F_1A	9.78	8.90	8.63	9.10±0.49
HPLC	F_1B	24.83	26.27	24.91	25.37±0.66
HPLC	F_1C	3.54	3.05	3.11	3.23±0.21
HPLC	F_1D	4.09	4.84	4.06	4.33±0.36
HPLC	F_1E	2.90	2.65	3.69	3.08±0.44

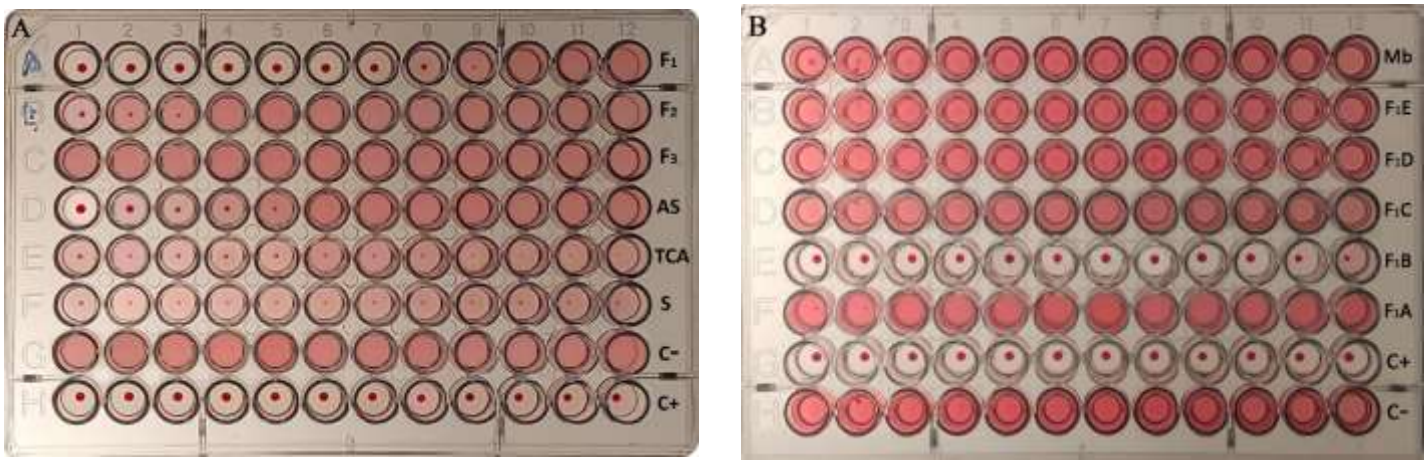

Figure 5. Complement fixation test for SEC fractions (**A**), and HPLC subfractions (**B**)
Mb: Brute mallein; **AS:** Precipitation with ammonium; **TCA:** Precipitation with trichloroacetic acid; **S:** Sonicated bacteria; **F_1, F_2, and F_3:** SEC fractions; **F_1A, to F_1E:** HPLC subfractions; C-: Negative control; **C+:** Positive control).

correlated with higher molecular weight proteins and exhibited more sensitivity in the mallein test (25).

Our findings agree with Verma et al. regarding the molecular weights of PPD-mallein proteins, although the two studies differ in the preparation methods.

This discrepancy can be attributed to the frequency (concentration gradient) of using AS; in fact, the separation of low weight proteins from the protein complex at multiple stages improved the results of the mallein test compared to other tests.

Da Silva et al. (2013) evaluated the efficacy of PPD-mallein in diagnosing glanders. They inoculated five suspected and five healthy equines with TCA- and AS-precipitated PPD. Only the suspected animals showed DHS reactions at the inoculation site, while healthy animals showed no inflammatory response, indicating the specificity of both methods (26). Their results support our findings that both TCA and AS methods induce DHS.

Until 2013, the PPD-mallein test was the primary diagnostic method recommended by WOAH for glanders detection, with the CFT and ELISA ranking second and third, respectively. However, the mallein test has gradually been considered less reliable due to potential veterinarian errors and the risk of cross-reaction with *B. pseudomallei*. Consequently, the CFT and ELISA have replaced the mallein test as the top two preferred tests (1).

Naureen et al. (2007) compared the efficacy of CFT and the PPD-mallein test in diagnosing glanders and reported better performance of CFT (27). Comparing this result with our findings suggests that combining multiple diagnostic tests can improve accuracy of glanders detection. This study suggests that the mallein test can be compared to CFT because of its superior performance with purified antigenic proteins. Consequently, depending on the laboratory and personnel conditions, each of these tests can be used to diagnose glanders confidently.

According to Pal et al. (2012), the efficiency of CFT decreases when whole-cell proteins are used directly. They improved the efficiency of CFT by purifying and producing antigenic proteins (12). Similarly, the purification of antigenic proteins in current study improved the results obtained from CFT, allowing reliable detection even at low dilutions.

Overally, the results demonstrated that the traditional PPD preparation method (using brute mallein- requires some modifications producing antigenic proteins through purification notably enhances diagnostic efficacy for glander. Moreover, purified antigenic proteins significantly improved the CFT results. Therefore, it can be concluded that the simultaneous use of the methods mentioned above and the optimization of the PPD preparation method, as explained in current study, can increase the efficiency or potency of the mallein test, allowing the more reliable reporting of positive cases of glanders and developmenta and implementation of more effective prevention and control measures.

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Authors' Contribution

Study concept and design: N. M.
Acquisition of data: M. B.
Analysis and interpretation of data: M. B N. M, M. B
Drafting of the manuscript: MB.
Critical revision of the manuscript for important intellectual content: N. M, M. F.
Statistical analysis: N. M
Administrative, technical, material support: N. M, M. F.

Ethics

All experimental procedures were approved by the animal care, and ethics committees of the Payame Noor University, Tehran, Iran (Accession number: IR.PNU.REC.1403.240)

Conflict of Interest

The authors declare that they have no conflicts of interest.

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Data Availability

The data supporting the findings of this study are available upon request from the corresponding author.

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