Isolation and purification of the *Burkholderia mallei* antigenic proteins and its use in diagnostic tests

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 the prevalence of glanders has caused a focus on control and eradication programs, emphasizing accurate diagnosis of infected cases using a high-performance test. To this end, antigenic proteins were purified from *Burkholderia mallei*, and the mallein test was then optimized using the 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 the proteins of 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 to compare the results. The protein profile of the samples was analyzed using SDS-PAGE. The mallein test was also performed, and the results were evaluated using CFT. The AS method was selected 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. The mallein test using the AS-participated proteins, the first fraction from SEC, and the second fraction from HPLC yielded significant results, demonstrating erythema with a diameter of 18.46, 21.70 and 25.37 mm, respectively. The mallein test results were confirmed by CFT. The findings suggested that the purified antigenic proteins improved the mallein test and CFT results. Consequently, these proteins can be used to diagnose glanders correctly and increase the accuracy of the mallein test and CFT.

Keywords: *Burkholderia mallei*, Glanders, Mallein test, Chromatography, Complement fixation test

1. Introduction

Glanders is a zoonosis and bacterial disease caused by *Burkholderia mallei*. This bacterium is responsible for transmitting dangerous and fatal infections in solipeds, particularly equines, due to the absence of effective and definitive treatments and the potential for aerosol transmission. The disease may also spread to humans through direct contact with infected animals, resulting in death in most cases. Therefore, the bacterium has the potential to be employed 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 improvement. The 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 the 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 has not been completely eliminated and occasionally appears as short epidemics; therefore, Iran can be considered one of the foremost centers of glanders globally (5).

The recent rise in the prevalence of glanders in the Middle East, the emergence of new epidemics, the ease of disease transmission, the absence of an effective vaccine for preventing glanders, and the potential use of the pathogen as a biological weapon make this disease a serious threat to the global health system (6,7). The prompt and accurate diagnosis of glanders

is of utmost significance. The international community needs access to information about the infected cases and their proper diagnosis in Iran. Hence, there is a growing demand for extensive studies in this regard and the utilization of advanced diagnostic tests to address the crisis caused by glanders effectively.

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 the mallein test causes different delayed hypersensitivity (DHS) levels, necessitating optimizing the mallein test by selecting effective (antigenic) proteins associated with the disease.

Moreover, the mallein test involves injecting a substance into the lower eyelid of an equine's eye; 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 task with a significant potential for errors and should be carried out solely by a qualified veterinarian. As a result, replacing the mallein test with other tests, such as CFT, is necessary to ensure that all pertinent procedures are carried out within the laboratory setting, allowing for effective control and mitigating potential errors (9-11).

Recently, some studies have been conducted on the occurrence and treatment of diseases based on their antigenic proteins. Such an approach can also be used to evaluate glanders (12,13). The efficacy of the mallein test will be enhanced by purifying the antigenic proteins of *B. mallei* and preparing PPD using these proteins. In addition, the use of antigenic proteins in CFT as a complementary or alternative test can yield more acceptable results, thereby contributing to reporting positive cases with greater confidence.

This study aimed to improve the mallein test results using purified antigenic proteins. Subsequently, the efficacy of purified PPD 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 of *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% of glycerol. Then the grown bacteria were inactivated at 70°C for 30 min (14). Bacteria were cultured on blood agar culture medium to ensure inactivation of the strains.

2.2. Cell Disruption

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 min at 4°C, then washed thrice with ice-cold phosphate buffered saline (0.01 M PBS, pH 7.0). The pellet was resuspended in PBS containing 1 mM phenylmethane sulfonyl fluoride and sonicated on ice at 90 amplitude for 5 cycles of 1 min pulsing with 1 min rests between the pulses (Hielscher-Ultrasound Technology, PN-66-NNN, Germany) then centrifuged at 9,000 g for 10 min. 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 Buchner funnel to strain the cell debris and 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 solution obtained from sonication was divided into two equal volumes. The proteins of each volume was precipitated by trichloroacetic acid (TCA) and ammonium sulfate (AS), respectively.

TCA precipitation. The sonicated mixture was mixed with 40% TCA at a ratio of 1:9 and stirred at 4°C for 6 h at 400 rpm. The mixture was then left overnight at 4°C without stirring. The precipitate was centrifuged at 2500 g for 15 min 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 a pH of 6.9±0.1 (16).

AS precipitation. AS gradually added to the sonicated mixture with stirring, in a 3 h period to reach 80% salt saturation at 4 °C. The solution was stirred for 6 h at 400 rpm and left at the same temperature for 12 h without stirring. The precipitate was centrifuged at 2500 g for 15 min after removing the supernatant the following day. The pellet subsequently dissolved in phosphate buffer (PBS; 6.9±0.1). The protein solution was dialyzed against 10 times distilled water for two days and then concentrated with polyethylene glycol (PEG) 6000 to a tenth of the initial volume (16).

2.5. Brute mallein preparation

Strain 325 of *B. mallei* was cultured on a Nutrient agar medium with 4% glycerol. After 48 h, the grown colonies were transferred to a 10 L beaker containing Bouillon medium with 4% glycerol and incubated for 30 days at 37°C. The grown bacteria were inactivated at 100°C for 1 h. 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. Then, phenol was added at a final concentration of 4% (17).

2.6. Potency assay

The precipitated proteins and brute mallein were tested for its potency based on six point assay, by sensitizing guinea pig models according to world organisation for animal health (WOAH) recommendations (1,18). Nine male outbred guinea pigs weighing 450-550 g, obtained from RVSRI, animal care unit were divided into three groups of three each. 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. The 0.01 mg/mL suspension was injected intramuscularly into the guinea pigs with a dosage of 0.5 mL three times at intervals of two weeks (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 h. The diameters of above 8 mm were regarded as positive reaction.

2.7. Complement fixation test (CFT)

CFT was carried out with the standard method as described by the WOAH (1,19). 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 control), were incubated at 56ºC for 30 min and 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 min. After adding 50 µL of the hemolytic system to each well, the plate was centrifuged for 1 min at 600 g and incubated for 30 min at 37ºC. The interpretation was 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. The resulting proteins were then purified in two chromatography steps.

Size-exclusion chromatography (SEC). The protein solution was sterilized by 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 a flow rate of 6 mL/h at 4°C and and its absorbances were recorded at 280 nm (20).

High-performance liquid chromatography (HPLC). Fractions obtained from SEC (100 µL injection volume) were analysed 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 was centrifuged at 2800 g to clear 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 a flow rate of 0.3 ml/min during 50 min. The fractions were monitored at 280 nm (21,22).

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 solution 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 (23,24).

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 using Coomassie blue and silver nitrate (25).

3. Results

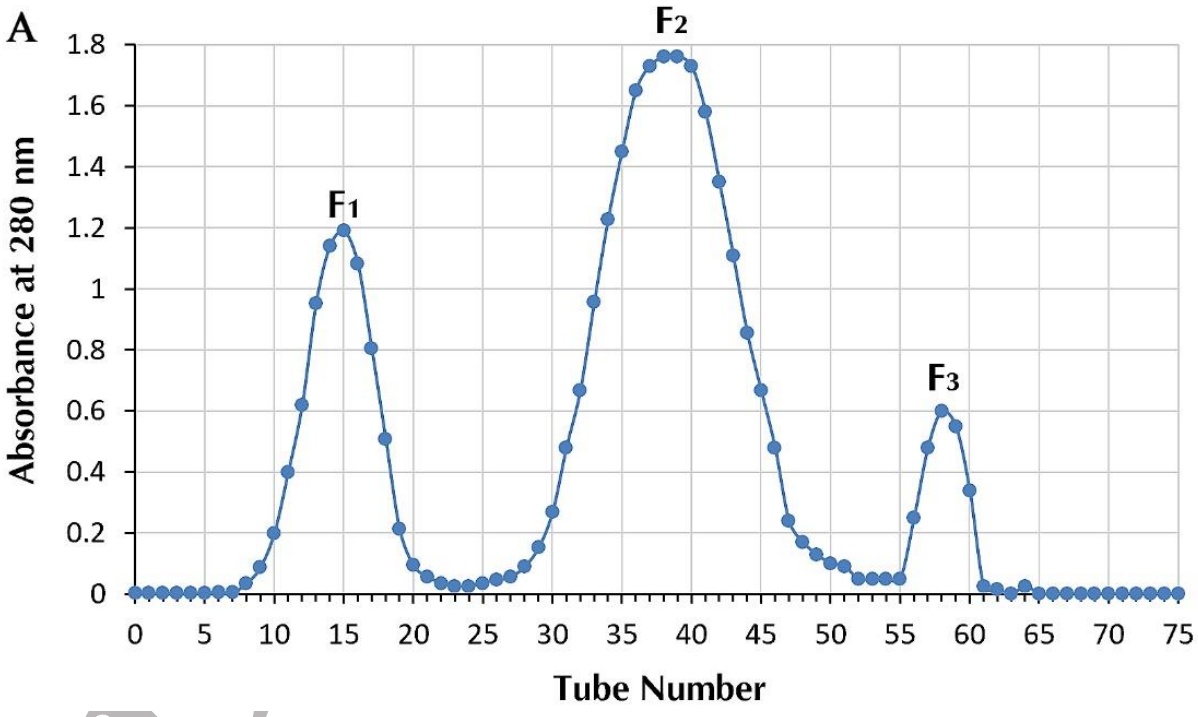
3.1. Bacterial culture and precipitation

After 48 h, *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 then dialyzed (Figure 1D).

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**)

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.



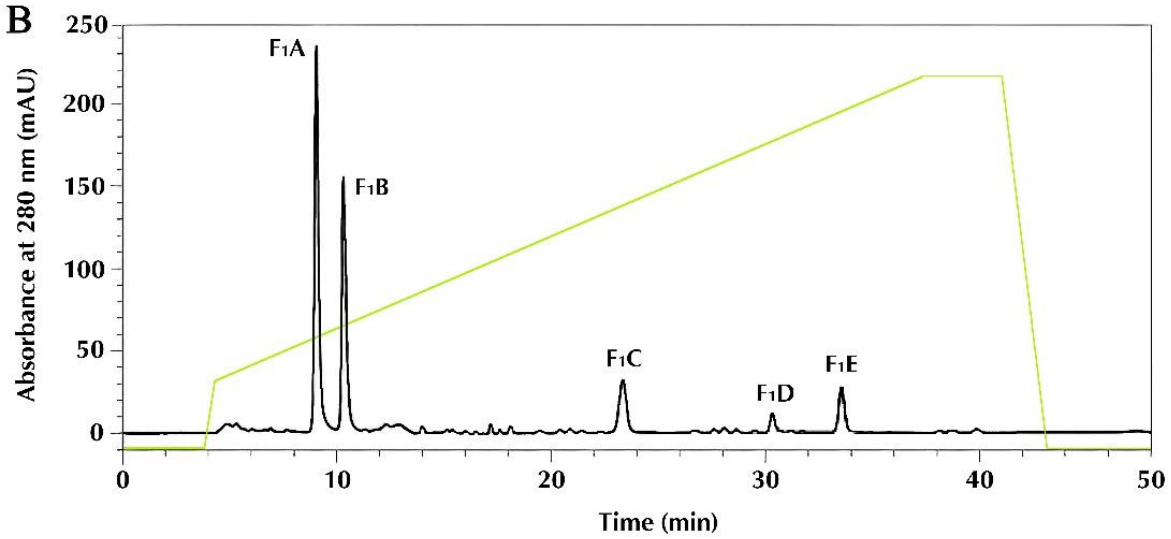


Figure 2. Chromatogram obtained from SEC (**A**) and HPLC (**B**)

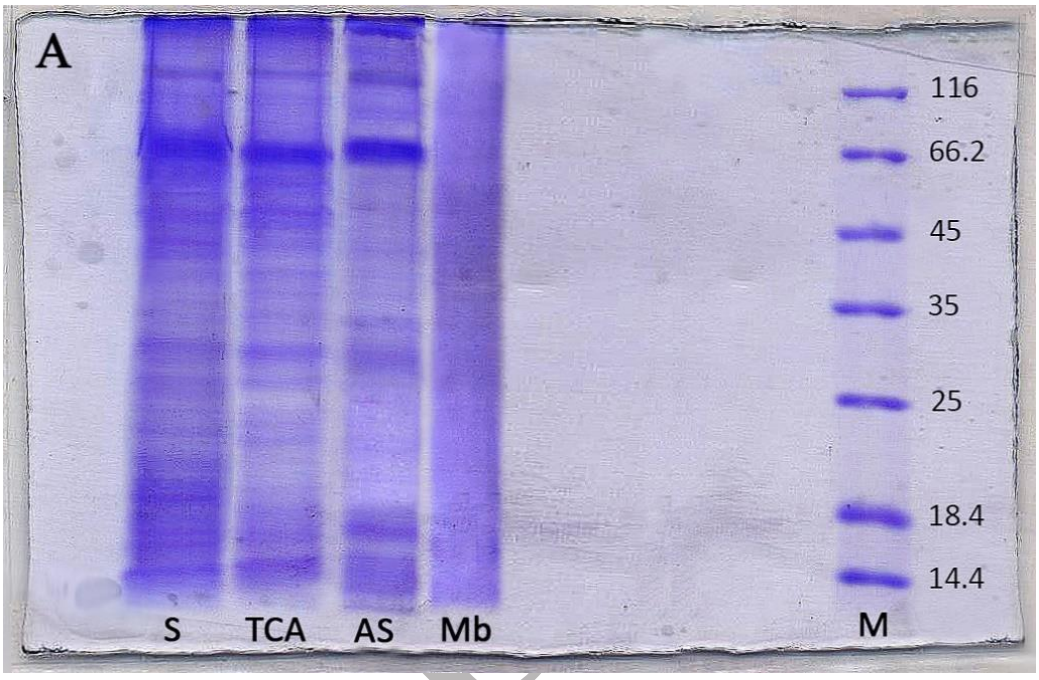
3.3. Protein assay

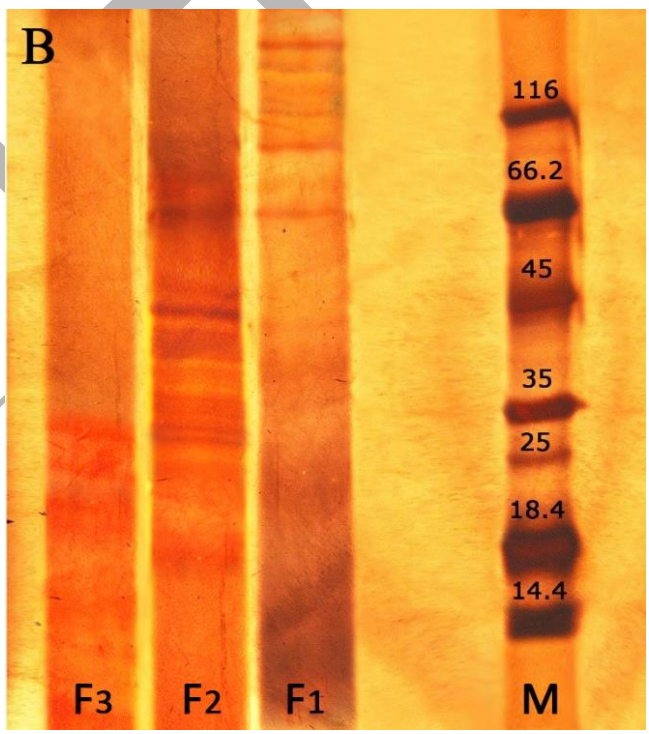
The protein concentrations of the samples, including the TCA-precipitated solution, concentrated AS, and brute mallein, were determined using the Kjeldahl method. The values obtained were 4.63, 2.92, and 2.78 mg/mL, respectively. After concentrating, the protein concentration of the F_1, F_2 and F_3 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 sonicated bacteria's protein and TCA-precipitated protein profile exhibited a significant abundance of a wide range of low to high molecular weight proteins. This profile was obtained as a smear through brute mallein. The AS-precipitated revealed the presence of a complex of low to high-molecular-weight proteins, albeit in reduced quantities (Figure 3A). The results showed the presence of proteins with a

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). The 40, 50, and 66 kD proteins were detected in F_1A and F_1B. In other sub-fractions, the protein concentration was so low that an SDS-PAGE band was not obtained. The standard protein contained a band in the kD range of 66, also observed in F_1B (Figure 3C).





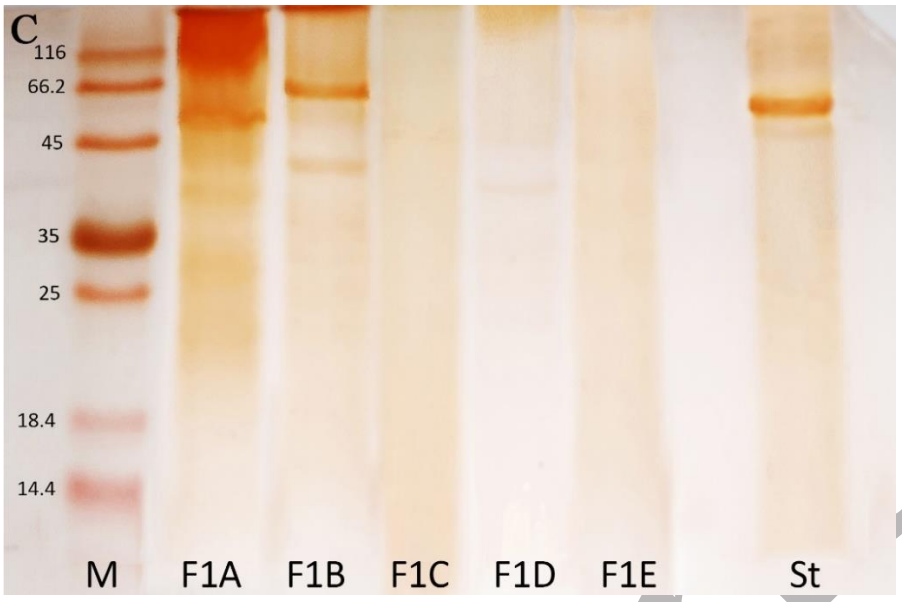


Figure 3. SDS-PAGE profile of precipitated proteins (**A**) and all fractions obtained from SEC (**B**) and HPLC (**C**)

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 with 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).

۲۴۴ **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

۲۴۵ *A diameter of 8 or less was considered non-reactive

۲۴۶ $P < 0.05$

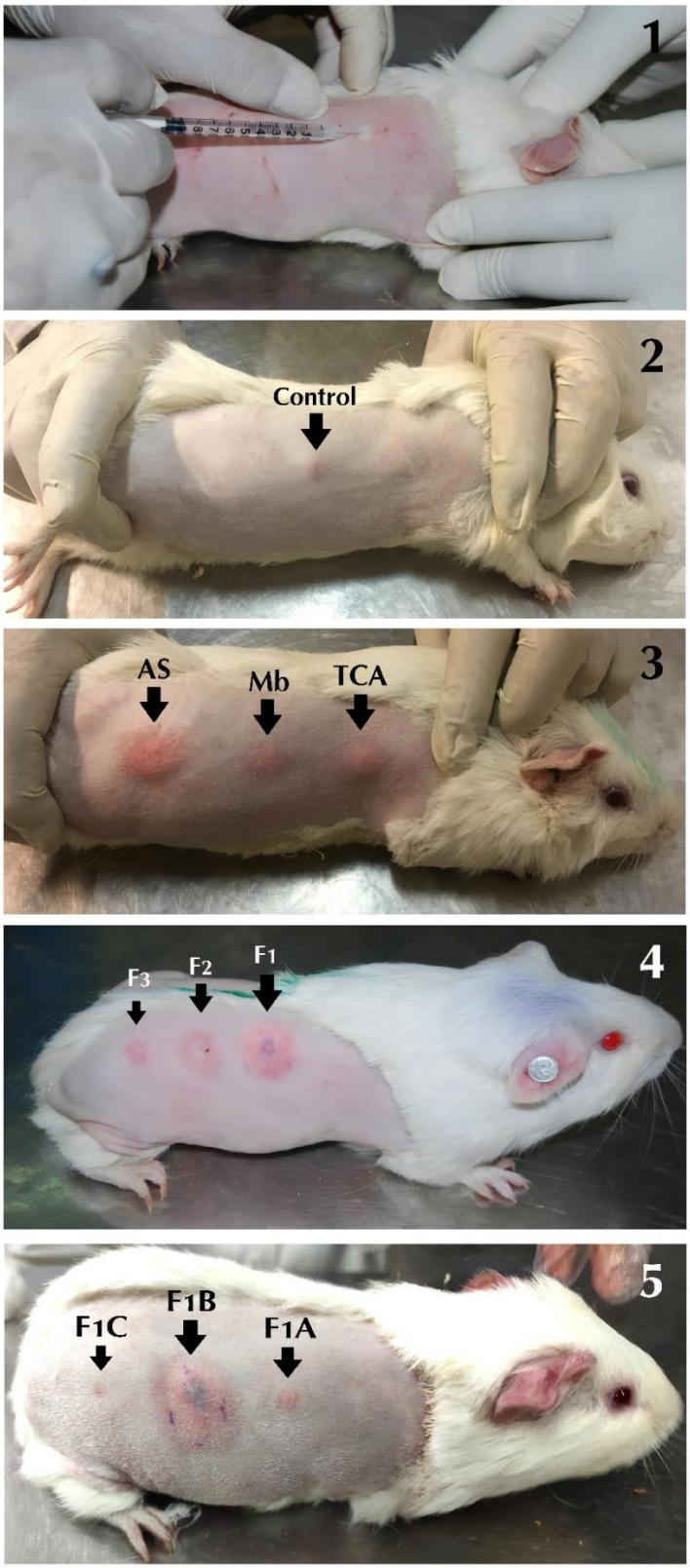
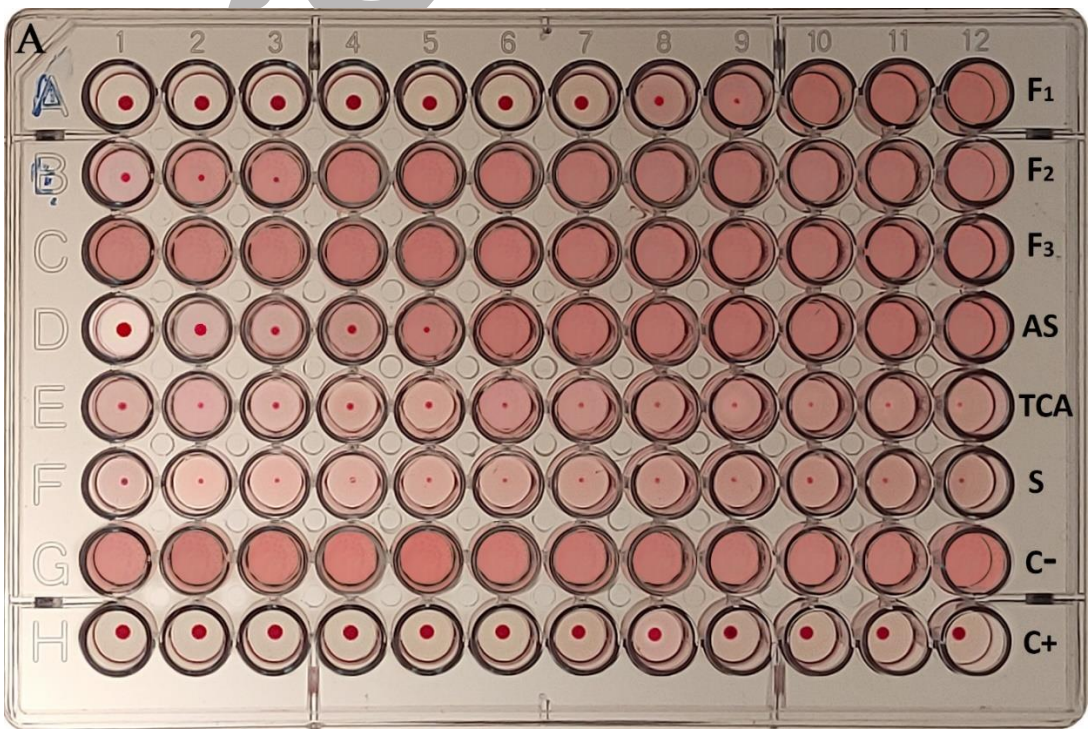


Figure 4. Mallein test [Injection of *Burkholderia mallei* proteins into sensitized guinea pig (**1**); Erythema developed in the control group (**2**); Mb, PPD_T, and PPD_A groups (**3**); SEC group (**4**) and HPLC group (**5**).

3.6. CFT

The initial CFT demonstrated that the AS-precipitated protein yielded superior outcomes, 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 instead showed varying degrees of hemoagglutination and hemolysis in 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 this well; F_2 exhibited hemoagglutination and hemolysis up to well 3, with complete hemolysis observed from well 3 onwards (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 exhibited superior performance compared to 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 well (at a 1:1 and 1:2 dilutions). However, except for F_1B, all other sub-fractions and brute mallein induced complete hemolysis (Figure 5B). Complete hemolysis and hemagglutination in the negative and positive control wells validated the accuracy of the CFT results (Figure 5 A and B).



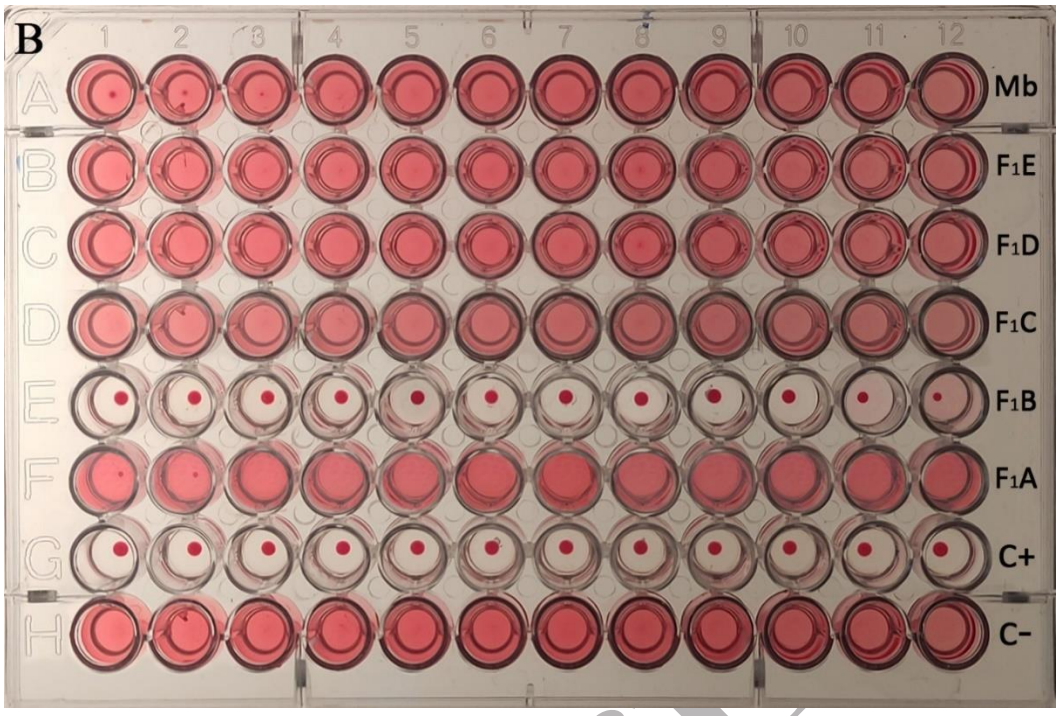


Figure 5. Complement fixation test for SEC fractions (**A**), and HPLC subfractions (**B**)

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)

4. Discussion

Glanders is a highly infectious and perilous bacterial disease that persists to be documented in solipeds and occasionally in 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. As a result, Iran is recognized as a stable endemic center for glanders, along with other countries in the Middle East. Because of the potential entry of unauthorized solipeds to Iran, it is crucial to identify glanders cases (7).

WOAH recommendations prioritize the production of PPD-mallein (1). In Iran, the mallein test is currently conducted using brute mallein. This protein complex is associated with varying levels of DHS. The variability of the mallein test may be attributed to the inactivation of the

bacteria at high temperatures or the prolonged heating of the protein composition to concentrate the mallein solution. This heating process causes the proteins to denature and significantly reduce their biological activity. Analyzing the SDS-PAGE profile in this study showed that all the proteins were in a denatured and smeared (Figure 3). To more accurately identify infected cases, optimizing this test to remove its variable results and produce a test with a high DHS was necessary.

The potency assessment results showed that protein precipitation with AS was a more useful method with better results. By comparing the results of AS with TCA methods, both performed under the same conditions, it is evident that the AS method is more effective. The lower potency of the TCA method can be attributed to the fact that it denatures the protein structure, resulting in removing bacterial carbohydrate and lipids or their binding to proteins in most cases.

In conjunction with 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 (glycoproteins and lipoproteins) from the PPD-mallein product.

In contrast, brute mallein and precipitation with TCA generate a substantial quantity of proteins (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. PPD-mallein was isolated from *B. mallei* by precipitating bacterial proteins with TCA and AS, followed by purification using gel filtration chromatography. TCA-precipitated proteins of relative purity exhibited the same potency and innocuity as the standard PPD-mallein. In contrast, AS-precipitated proteins exhibited a nonspecific reaction. Further investigations demonstrated that the activity of PPD-mallein was associated with higher molecular weight proteins and exhibited more sensitivity to the mallein test (26). The results of current study were consistent with the findings of Verma et al. (1994) regarding the molecular weight of PPD-mallein proteins; nevertheless, the two studies reported different results in the PPD-mallein preparation method. 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 effectiveness of PPD-mallein in the diagnosis of glanders. To this end, five equines suspected of glanders and five healthy equines were treated with TCA- and AS-precipitated PPD. Healthy equines showed no inflammatory reaction at the inoculation site, whereas the suspected equines exhibited a DHS allergic reaction as an inflammatory response (27). Their results are consistent with the findings of current study regarding the induction of DHS by both TCA and AS methods.

Until 2013, the PPD-mallein test was the primary diagnostic test recommended by WOAH for detecting glanders, while the CFT and ELISA ranked second and third, respectively. However, the mallein test has been gradually considered less reliable due to potential errors by the veterinarian and the risk of cross-reaction with *B. pseudomallei*. As a result, the CFT and ELISA supplanted the mallein test as the top two choices (1).

Naureen et al. (2007) compared the efficacy of CFT and the PPD-mallein test in diagnosing glanders and reported better performance of CFT in diagnosing glanders (28). Comparing this result with our findings reveals that glanders can be more effectively diagnosed using several diagnostic tests simultaneously. 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), using whole-cell proteins reduces the efficiency of tests like CFT. Therefore, 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, as it produced satisfactory results even at low dilutions.

The study results demonstrated that the traditional PPD preparation method (with brute mallein) needs some modifications. Accordingly, the optimization of this method by producing antigenic proteins substantially improved the efficacy of glander diagnosis. 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 the developmenta and implementation of more effective prevention and control measures.

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Authors' Contributions

Conceptualization: NM

Data curation: MB

Investigation: MB

Methodology: NM, MB

Project administration: NM, MF

Supervision: NM

Writing–original draft: MB

Writing–review & editing: NM, MF

Conflict of Interest

The authors declare that they have no conflicts of interest.

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)

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Data availability

The data that support the findings of this study are available on request from the corresponding author.

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