Original Article

Identification of *Burkholderia mallei* Isolates with Polymerase Chain Reaction-Restriction Fragment Length Polymorphism

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ABSTRACT

Burkholderia mallei is the main cause of glanders as a dangerous contagious zoonosis disease that is mostly observed in single-hoofed animals, especially horses. Modern molecular techniques have been recently employed to improve epidemiology for identifying and searching for strains of this bacterium at different times and locations. Due to the unknown number of circulating strains and lack of preventive methods, glanders is still observed in the form of epidemics. The present study aimed to evaluate six field isolates plus two laboratory strains of *Borkolderia mallei* and *Burkholderia pseudomallei* using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. All the isolates and strains were microbially cultured in the glycerol nutrient and glycerol agar media. The individually grown colonies of the bacterium were used in the biochemical tests. The DNA of isolates was extracted by boiling, and the PCR-RFLP test was conducted on their genome. Finally, the bacterium was injected into guinea pigs to induce the Straus reaction. The biochemical assays (or bioassays) confirmed the isolates as *Burkholderia mallei.* The PCR-RFLP assay demonstrated a product for *Burkholderia mallei* with a length of 650 bp. Nevertheless, 250 and 400 bp were produced for *Burkholderia pseudomallei.* The swollen scrotum pointed to the occurrence of the Straus reaction. The PCR-RFLP is a proper differential diagnosis technique for *B. mallei;* moreover, it is a suitable method for differentiating between *Burkholderia mallei* and *Burkholderia pseudomallei*. This technique can detect *Burkholderia mallei* in a short time with high precision and sensitivity.

Keywords: *Borkolderia mallei*, Glanders, PCR-RFLP

1. Introduction

Glanders is among the oldest known diseases caused by the gram-negative bacterium B. *mallei* which is a gram-negative, aerobic, nonmotile, non-capsulated, and non-spore-forming bacillus that naturally causes disease in horses, mules, and donkeys. Although this disease can infect other species, it is mainly observed as a hidden chronic disease in horses. The disease occurs in humans accidentally and in felines and Canidae via the consumption of infected meat (1).

In recent centuries, glanders has been among the most prevalent diseases in single-hoofed animals across the globe. In the middle of the 20th Century, this disease was eradicated in most countries through quarantine and control measures, such as the identification and extermination of infected animals. The rate of infection was very high in Iran in 1919; nonetheless, the last glanders endemics in Iran occurred in 1973. Since then a few cases of animal or human infection with glanders have been reported in Iran (2).

The sporadic outbreak of this disease in Iran can be attributed to increased breading and maintenance of horses and illegal transport of livestock. The mortality rate increases under such circumstances so that apparently healthy animals are not fully treated and the transport of infected horses may cause the recurrence of glanders (3). Due to the cross-reactivity of serological tests for *B. mallei* and *B. pseudomallei*, it is not possible to exactly evaluate the global spread of glanders. There is neither a definitive treatment for animals with glanders nor an effective vaccine to prevent this disease. Therefore, the identification of infected animals is the most effective method to prevent the spread of glanders (4, 5).

Due to similar clinical symptoms of glanders with some other diseases, it is usually difficult to diagnose this disease based on its clinical symptoms (6). The mallein test which is currently used in disease control programs for the diagnosis of glanders in animals lacks high sensitivity in humans; moreover, due to low specificity, it cannot differentiate *B. mallei* from *B. pseudomallei*. Glanders can be definitively diagnosed by positive culture of *B. mallei* (7, 8).

In cases where the tests wrongly diagnose *B. mallei* rather than *B. pseudomallei*, molecular biology, such as the 16s ribosomal RNA gene sequence analysis, or specific polymerase chain reaction (PCR) assays for *B. mallei* may be required to confirm the diagnosis (9-11). According to tests in recent years, genotyping techniques are more suitable than phenotyping methods for epidemiological studies on *Burkholderia* isolates (12). The molecular methods used are mainly based on PCR and include various types, such as PCR-restriction fragment length polymorphism (PCR-RFLP) and variable number tandem repeat (VNTR) (13, 14).

The RFLP is the presence of heterogeneous patterns characterized by the effect of enzymatic digestion in a specific region of the *B. mallei* DNA by restriction enzymes. These heterogeneous patterns occur due to DNA differences depending on the presence or absence of restriction enzymes (10). To control and eradicate glanders, it is essential to identify the number of circulating strains, differentiate similar strains (*B. pseudomallei*), and study the geographical distribution (molecular epidemiology) of the cause of glanders. Glanders and the bacterium causing this disease should be seriously considered to manage the probable crisis caused by glanders. In light of the aforementioned issues, the present study aimed to evaluate six clinical isolates, as well as one standard *Borkolderia mallei* strain and one *Burkholderia pseudomallei* strain, using the PCR-RFLP method.

2. Materials and Methods

2.1. Isolates

The samples were taken from infected livestock from 2010-2017 by the assessment of six cases of glanders in Tehran Zoological Garden (Siberian tiger, 2010) in Alborz Province (2011), Oshnavieh in East Azerbaijan Province (2014), Semirom in Esfahan Province (2017), Qom, and Kermanshah provinces (2016). The samples were then transferred to the Razi Vaccine and Serum Research Institute. In 2021, all isolates were recovered

from an archive kept at -70°C, cultured in 1% glycerol nutrient medium, and incubated at 37°C for 48 h.

2.2. Bacterial Culture

The laboratory strain of *B. mallei* 325 (RTCC 2375), a *B. pseudomallei* strain (ATCC 23343), and isolates were cultured by conventional methods on specific glycerol nutrient and glycerol agar media (Merck, Germany). After 48 h of incubation at 37°C under aerobic conditions, the slides were prepared from colonies grown on the solid medium and then examined by gram staining (3).

2.3. Biochemical Experiments

Individual bacterial colonies grown on the glycerol nutrient medium were used in the Simmons citrate and SIM tests using the API20 kit (bioMérieux, Inc, USA) and the TSI and mobility tests using specific media (15). The laboratory *B. mallei* strain used for producing mallein in Iran was considered the positive control.

2.4. Molecular Experiments

2.4.1. Inactivation of Bacteria and Genomic DNA Extraction

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 (O-ring). 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 (bacterial genome content) 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 for any sign of probable bacterial growth. After ensuring the deactivation of bacteria, the suspensions containing the extracted bacterial genome were stored in a refrigerator or freezer for further use in the molecular assays (14).

2.4.2. PCR-RFLP

An identical specific primers pair of *B. mallei* and *B. pseudomallei* were used in the PCR-RFLP assay.

A PCR primer pair specifically designed by Tanpiboonsak and co-workers was selected (14) to amplify a fragment of 650 bp length from both *B. mallei* and *B. pseudomallei* was used. A final volume of 12 µL was set in the PCR reaction, including 6 µL of the master mix (Ampliqon, Denmark), 1 µL of the solution (5 pM/µL) 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 inverse primer, as well as 1.5 µL of the bacterial genome-containing suspension and 2.5 µL double distilled water. Double distilled water and *B. mallei* genome were respectively used as the negative and positive controls. The thermal protocol used for amplification by the thermocycler (Eppendorf, Germany) includes 5 min initial heating at 94°C, followed by 34 cycles, including 1 min heating at 94°C, 1 min heating at 68°C (to attach primers to the target site on the bacterial genome), 1 min heating at 72°C (for amplification), and eventually 10 min heating at 72°C (to complete the amplification process). For the enzymatic reaction after the amplification of the desired region, the PCR product was enzymatically digested by the *Bsp1431* (*Sau3AI*) enzyme. Electrophoresis was performed using Red Safe (Fentoskova, Russia) pre-stained 1% MP agarose (Roche, Germany) with a genetic marker with a size of 100 base pairs for 90 min at 2 V/cm (14).

2.5. Bacterial Injection into a Sensitive Host

In this test, eight male albino guinea pigs with an approximate weight of 300-400 g were used. From the new culture of *B. mallei* and six isolates, a bacterial suspension with turbidity equivalent to the McFarland Tube #1 (3×10^8 CFU/mL) was prepared in the physiological serum solution. The guinea pigs were transferred to the isolator, and 1 ml of the bacterial suspension was injected intraperitoneally

in seven guinea pigs (animal 1: *Borkholdria mallei* strain, animal 2: Siberian tiger isolate, animal 3: Kordan isolate, animal 4: Oshnavieh isolate, animal 5: Semirom isolate, animal 6: Qom isolate, animal 7: Kermanshah isolate, animal 8: physiological serum). After injection, the guinea pigs were constantly monitored for 72 h, and after inducing the Straus reaction, the infected guinea pigs were necropsied under sterilized conditions, and the samples were taken from testicles, liver, lung, and spleen in search for bacterial *B. mallei* culture (13, 16)

3. Results

The isolates were grown on a nutrient agar medium. The mucoid colonies were round, smooth, convex, and translucent in gray color (Figure 1). Microscopical examinations demonstrated gram-negative curved bacilli and coccobacilli with rounded sides.

Figure 1. Growth of *B. mallei* on nutrient agar medium. Most B. mallei strains formed circular, translucent, smooth, and > 1 mm colonies on nutrient agar medium

The results of biochemical assays (Figure 2) confirmed isolates as *B. mallei*. Nonetheless, the no change-no change mode, immobility, the lack of indole formation, and the lack of sugar consumption, respectively, in the TSI, mobility, SIM, and Simmons citrate media confirmed the presence of *B. mallei* (Table 1).

Figure 2. Biochemical experiments with APi20 kit (top: uninoculated, bottom inoculated)

Table 1. Biochemical results of *B. mallei* and other isolates

Medium / Strains	TSI	Motility	SIM	Citrate
Standard strain RTCC 2375	No Change/ No Change	No	Negative Indole	Negative
Isolates	No Change/ No Change	No	Negative Indole	Negative

A high-quality product was proliferated in the PCR-RFLP assay, and the genome DNA patterns of *B. mallei* digested by the *Sau34I* restriction enzyme illustrated a difference in the polymorphic patterns of the fragment length in 0.9-1.5 base pair fragments. The effect of the enzyme on *B. mallei* developed a product with a size of 650 base pairs, as well as 250 and 400 base pair fragments, for *B. pseudomallei* (Figure 3).

Swollen scrotum membranes in the *B. mallei* strain and the studied isolates pointed to the occurrence of the Straus phenomenon and bacterial growth in the scrotum membranes of guinea pigs (Figure 4).

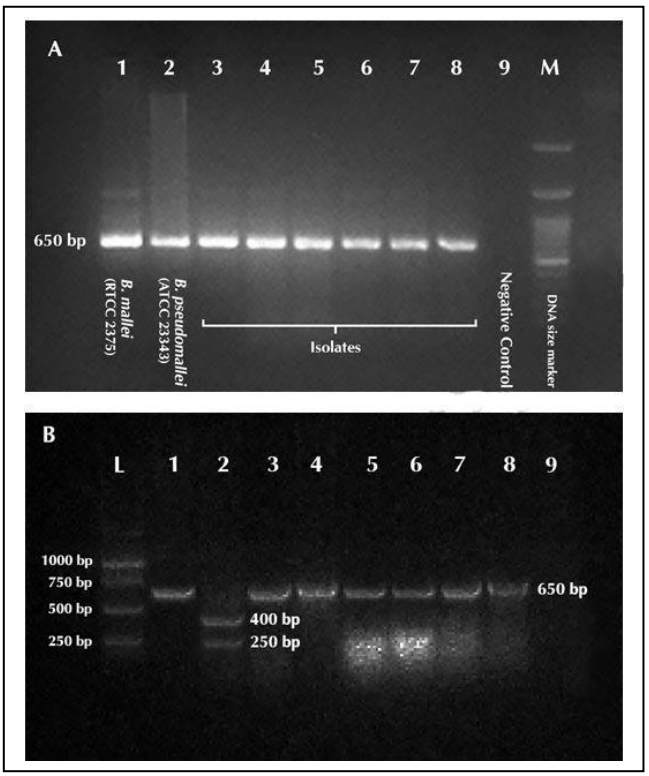

Figure 3. PCR-RFLP for the studied samples
M: DNA size marker; 1: *B. mallei* (RTCC 2375); 2: *B. pseudomallei* (ATCC 23343); 3-8: Isolates; 9: Negative control

Figure 4. Injection of *B. mallei* into guinea pig peritoneum (Strauss reaction). A small quantity of an emulsion of *B. mallei* was inoculated into the peritoneal cavity of a male guinea pig. Within three days, the testes became swollen and inflamed; subsequently, they were converted with a caseous mass to which the skin was adherent

4. Discussion

The *B. mallei* is the cause of glanders as one of the most dangerous contagious diseases which mainly affect monogamous animals. Glanders is a zoonosis used as a biological weapon; therefore, it is of paramount importance to investigate the different aspects of the responsible bacterium (6). The identification of infected animals is the most effective way to prevent the spread of glanders. In all cases, the occurrence of glanders should be immediately reported to health authorities. In the case of the detection of glanders, control measures should be taken, such as quarantining infected animals, diagnostic tests on animals with suspicious symptoms, as well as evaluating healthy animals and separating them in the case of a positive mallein test (17).

Various types of biological weapons have been produced and sometimes used by most industrial countries, especially Russia, the USA, the United Kingdom, France, Japan, Germany, and Canada. The *B. mallei* have been used by Russia, the United Kingdom, France, Germany, and Japan as biological weapons (17). Accordingly, all strains in Iran should be identified and compared with those in neighboring countries and other regions around the world to rapidly identify and cope with new strains or biological attacks.

The genetic structure of *B. mallei* has been extensively studied by molecular techniques. It is, therefore, necessary to use reliable PCR techniques to definitely specify the genome of the disease cause and precisely and rapidly diagnose glanders. According to the obtained results, the PCR-RFLP method used in the present study suffices for identifying the cause of *B. mallei* (14, 18).

The results of the current research pointed out that the cause of glanders can be detected rapidly by PCR techniques without any need for a culture medium. This is of great importance for the identification of epidemics and probable risks of microbial attacks. In addition to differentiating *Burkholderia* species from similar species, such as *Pseudomonas*, *B. mallei* species can be detected from similar species, (i.e., *B. pseudomallei*) which is of utmost importance in the rapid differentiation of these two species.

Tanpiboonsak, Paemanee (14) identified and differentiated between *B. mallei* and *B. pseudomallei* by the PCR-RFLP assay. According to the results of their study, the PCR-RFLP test can identify these two species and differentiate *B. mallei* from *B. pseudomallei*. The PCR-RFLP technique detected a 650 base pair fragment of *B. mallei*, whereas two fragments of PCR-RFLP products were obtained, including 250 and 400 base pairs of *B. pseudomallei* (14). The results of the study by Tanpiboonsak, Paemanee (14) were in line with the findings of the current research.

The specific gene has been considered a useful genetic marker for studying diversity and significance in pathogenesis, as well as variation, within a population of closely related bacteria (19). Since *B. mallei* is the sole species in this genus which is non-motile, the specific gene would be the best target for PCR-based differentiation of the species from others in the genus *Burkholderia*.

Furthermore, the 16S rDNA of the two species are identical. It is obvious that the DNA sequences related to gene evolution are highly conserved in these two organisms. In an attempt to find a better genetic marker for the differentiation between the two species, it is suggested that the use of other techniques, along with PCR-RFLP, may improve the diagnostic value of this method. In the achievement of a more thorough understanding of *B. mallei* detection and glanders epidemiology, such techniques as VNTR, pulsed-field gel electrophoresis (PFGE), and Southern blot hybridization, can enhance the quality of implementation and principles of the disease control program.

The PCR amplification products of about 650 bp were detected from both species. As evidenced by the obtained results, both organisms contain the same DNA sequences, except for a one-point mutation in the *B. pseudomallei* sequence which was recognized by *Sau3AI* restriction endonuclease. The PCR-RFLP based

on the restriction enzyme *Sau32AI* is a high-differentiation typing method for the diagnosis of *B. mallei*; moreover, it is useful to differentiate between the *B. mallei* and *B. pseudomallei* species. This method can detect *B. mallei* within a short time with high sensitivity. Accordingly, this specific marker is a selective method to ensure the identification of these two species.

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Authors' Contribution

Study concept and design: N. M.
Performed the experiments: F. A.
Analysis and interpretation of data: N. M., S. A. P., K. T., M. J.
Drafting of the manuscript: F. A.

Ethics

We hereby declare that all ethical standards have been respected in the preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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