Identification of *Burkholderia mallei* isolates with PCR-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 seen in monogamous animals, especially in horses. Modern molecular techniques have been recently employed to improve epidemiology for identifying and searching for strains of this bacterium in different times and locations. Due to the unknown number of circulating strains and the lack of prevention of glanders, the disease is still observed in the form of short-term and point epidemics. The aim of this study was to evaluate six clinical isolates, one standard *Borkolderia mallei* strain and one *Burkholderia pseudomallei* strain using PCR-Restriction fragment length polymorphism (PCR-RFLP) method. Standard isolates and strains of *Burkholderia pseudomallei* 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. Eventually, the bacterium was injected to guinea pigs to induce the Straus reaction. The biochemical assays (or bioassays) confirmed the isolates as...
*Burkholderia pseudomallei*. The PCR-RFLP assay showed a product for *Burkholderia mallei* with a length of 650 bp. However, 250 and 400 bp were produced for *Burkholderia pseudomallei*. The swollen scrotum was suggestive of the occurrence of the Straus reaction. PCR-RFLP is a proper differential diagnosis technique for *B. mallei*. It is also a suitable method for differentiating *Burkholderia mallei* and *Burkholderia pseudomallei*. This technique detects *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*. This is a gram-negative, aerobic, nonmotile, non-capsulated, non-spore-forming bacillus that naturally causes disease in horses, mules, and donkeys. Even though it also infects other species, it is mainly observed as a hidden chronic disease in horses. The disease occurs in humans accidentally and in felines and Canidae by eating infected meat (1).

In recent centuries, glanders has been among the most prevalent diseases in monogamous animals around the world. In the middle of the 20th Century, the disease was eliminated through quarantine and control measures in most countries such as identifying and exterminating infected animals. The rate of infection was very high in Iran in 1919 but the last glanders endemics occurred in 1973 in Iran. Afterward, a few cases of animal or human infection with glanders have been reported in Iran (2).

The outbreak of the disease in Iran is probably due 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 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 no definitive treatment for animals with glanders and also an effective vaccine to prevent the disease. Thus, identifying infected animals is the most effective method to prevent the spread of glanders (4,5).

Due to similar clinical symptoms of glanders with some diseases, it is usually difficult to diagnose this disease from 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 is 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 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-RFLP and Variable number tandem repeat (VNTR) (13,14).

Restriction fragment length polymorphism (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. Like developed countries, glanders and
the bacterium causing this disease should be seriously considered to manage the probable crisis caused by glanders. The aim of this study was to evaluate six clinical isolates, one standard *Borkholderia mallei* strain and one *Burkholderia pseudomallei* strain using PCR-RFLP method.

2. Materials and Methods

2.1. Isolates

Samples were taken from infected livestock from 2010 to 2017 by studying 6 cases of glanders in the Tehran Zoological Garden (Siberian tiger, 2010), Kordan 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 and cultured in the 1% glycerol nutrient medium, and then incubated at 37°C for 48 h.

2.2. Bacterial culture

The standard *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 (Merek, Germany). After 48 h of incubation at 37°C under aerobic conditions, slides were prepared from colonies grown on the solid medium and then examined by gram staining (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 in terms of any sign of probable bacterial growth. After ensuring the deactivation of bacteria, suspensions containing the extracted bacterial genome were stored in a refrigerator or freezer until being used in the molecular assays (14).

2.4.2. PCR-RFLP

An identical specific primers pair of B. mallei and B. pseudomallei were used in the PCR-RFLP assay. The target gene for specific primers of B. mallei and B. pseudomallei such as Tanpiboonsak et al. was selected (14). 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, 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 (for completing the amplification process). For the enzymatic reaction after amplification of the desired region, the PCR product was enzymatically digested by the Bsp143I (Sau3AI) enzyme. Electrophoresis was performed using Red Safe (Fentoskova, Russia) pre-stained 1% MP agarose (Roche, Germany) with a genetic marker size of 100 base pairs for 90 min at 2 V/cm (14).

2.5. Bacterial injection into a sensitive host

In this test, 8 male albino guinea pigs with an approximate weight of 300 to 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 (3x10^8 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 7 groups of pigs (Group 1: Borkholdria mallei strain, Group 2: Siberian tiger isolate, Group 3: Kordan isolate, Group 4: Oshnavieh isolate, Group 5: Semirom isolate, Group 6: Qom isolate, Group 7: Kermanshah isolate, Group 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 samples were taken from testicles, liver, lung, and spleen for bacterial B. mallei culture (13,16).

3. Results
The isolates were grown on nutrient agar medium. The mucoid colonies were round, smooth, convex, and translucent in gray color (Figure 1). Microscopical examinations showed 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*. However, 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).
Table 1. Biochemical results of \textit{B. mallei} and other isolates

<table>
<thead>
<tr>
<th>Medium Strains</th>
<th>TSI</th>
<th>Motility</th>
<th>SIM</th>
<th>Citrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard strain RTCC 2375</td>
<td>No Change/ No Change</td>
<td>No</td>
<td>Negative Indole</td>
<td>Negative</td>
</tr>
<tr>
<td>Isolates</td>
<td>No Change/ No Change</td>
<td>No</td>
<td>Negative Indole</td>
<td>Negative</td>
</tr>
</tbody>
</table>

A high-quality product was proliferated in the PCR-RFLP assay, and the genome DNA patterns of \textit{B. mallei} digested by the \textit{Sau34I} restriction enzyme showed a difference in the polymorphic patterns of the fragment length in 0.9-1.5 base pair fragments. The effect of the enzyme on \textit{B. mallei} produced a product with a size of 650 base pairs and 250 and 400 base pair fragments for \textit{B. pseudomallei} (Figure 3).
M: DNA size marker; 1: *B. mallei* (RTCC 2375); 2: *B. pseudomallei* (ATCC 23343); 3-8: Isolates; 9: Negative control

Swollen scrotum membranes in the *B. mallei* strain and the studied isolates indicated the occurrence of the Straus phenomenon and bacterial growth in the scrotum membranes of guinea pigs (Figure 4).
Figure 4. Injection of *B. mallei* into guinea pig peritoneum (Strauss reaction). 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

*B. mallei* is the cause of glanders as one of the most dangerous contagious diseases mainly in monogamous animals. Glanders is a zoonosis with a high potential to being used as a biological weapon for several reasons indicating the need for investigating different aspects of this bacterium (6). Identifying infected animals is the most important way to prevent the spread of glanders. In all cases, the occurrence of glanders should be immediately reported to health authorities. In the case where glanders is detected, control measures should be taken such as quarantining infected animals, diagnostic tests on animals with suspicious symptoms, and 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, in particular Russia, USA, United Kingdom, France, Japan, Germany,
and Canada. *B. mallei* have been used by Russia, United Kingdom, France, Germany, and Japan as biological weapons (17). Accordingly, all strains in Iran and around the world should be identified and compared with strains 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 results, the PCR-RFLP method used in this study suffices for identifying the cause of *B. mallei* (14, 18).

The results showed 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 identifying 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 great importance in the rapid differentiation of these two species.

Tanpiboonsak *et al.* (2004) identified and differentiated *B. mallei* and *B. pseudomallei* by the PCR-RFLP assay. According to their results, 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 Tanpiboonsak *et al.* were consistent with the results of this study.

The specific gene has been considered 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 therefore would be the best target for PCR-based differentiation of the species from others in the genus *Burkholderia*. 
In addition, the 16S rDNA of the two species are identical. Obviously, the DNA sequences related to the gene evolution are highly conserved in these two organisms. In an attempt to find a better genetic marker for differentiation of the two species, therefore, we would suggest that the use of other techniques along with PCR-RFLP may improve the diagnostic value of this method. While achieving a more correct understanding of \textit{B. mallei} detection and glanders epidemiology, techniques such as VNTR, PFGE and Southern blot hybridization can enhance the quality of implementation and principles of the disease control program.

**Conclusion**

The PCR amplification products of about 650 bp were detected from both species. The result indicated that both organisms contain the same DNA sequences excepting a one-point mutation in the \textit{B. pseudomallei} sequence which was recognized by \textit{Sau3AI} restriction endonuclease.

PCR-RFLP based on the restriction enzyme \textit{Sau3AI} is a high-differentiation typing method for the diagnosis of \textit{B. mallei}. It is useful to differentiate the \textit{B. mallei} and \textit{B. pseudomallei} species. This method can detect \textit{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.

**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
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Ethics

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

Consent for publication

All the authors consent to publish the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

References


