

Genomic pattern analysis of *Burkholderia mallei* field isolates by pulsed-field gel electrophoresis (PFGE) discriminatory typing

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ABSTRACT

Background and Objectives: Glanders is a serious zoonotic disease caused by *Burkholderia mallei*. Prevention, control, and treatment strategies of glanders are prerequisites for microbial source tracking. The present study was aimed to analyze the genomic pattern of *B. mallei* Iranian field isolates by pulsed-field gel electrophoresis (PFGE) typing.

Materials and Methods: *B. mallei* isolates were aerobically cultured in nutrient broth/agar supplemented with glycerol 4% for 48 h at 37°C. API 20NE identification system was used for the biochemical characterization. Genomic DNA of bacterial isolates was extracted using OIE-recommended protocol. Molecular identification of bacterial isolates was done based on amplification of *BimA* and IS407-flip genes. PFGE was applied to prepare the genomic pattern of *B. mallei* isolates. The guinea pig was used as a suitable model for studying the histopathological characterization of *B. mallei*.

Results: In both enzymatic digestion patterns by using *AflIII* and *VspI*, we found three different clonal types; I) PFGE type of *B. mallei* Razi 325 strain, II) PFGE type of Tiger, Kordan, and Oshnavieh strains, and III) PFGE type of Semiroom strain. *B. mallei* Razi 325 was categorized as unrelated strain which was belonged to the different cluster differing more than four bands.

Conclusion: PFGE showed more discriminatory power and considerable reproducibility for molecular typing of *B. mallei* strains in our study. It is standardized the approaches for outbreak detection, pathogen phylogeny, molecular epidemiology, and population studies.

Keywords: *Burkholderia mallei*; Pulsed-field gel electrophoresis; Glanders; Zoonoses; Biological warfare

INTRODUCTION

Burkholderia mallei, a Gram-negative and facultative intracellular coccobacillus is the causative agent of glanders, a serious pulmonary disease of solumpeds (i.e., horses, mules, and donkeys) endemic to

Asia, the Middle East, Africa, and Central and South America (1). Equine is the preferred host for glanders and it is largely due to ingestion of feed or water contaminated with nasal discharges from infected animals. Moreover, the infection has been reported in other animals following the consumption of glander-

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ous horses (2). The manifestation of glanders is usually chronic in the horse, while in mule, zebra, and donkey the disease is acute (3). Glanders is known as a zoonotic disease predominantly affecting Equidae and infrequently human beings through the occupational exposure and biological warfare (BW) (4). *B. mallei*, a known biothreat agent, has been listed as the Category B Potential Bioterrorism agent by the Centers for Disease Control and Prevention (CDC) (5).

Iran is on the unintended path of regional glanders due to the existence of geographical and economical commonalities with the neighboring countries possessing endemic diseases and consequently, they have been strengthening the source tracking on *B. mallei* Iranian strains. Furthermore, the weakness of neighboring countries in controlling and preventing the diseases and the passage of the pack animals, beast of burden, at the country's borders have made Iran the major center of glanders in the world (6).

Recently, sporadic reports of the glanders in the western provinces of Iran, loss of a male Siberian (or Amur) tiger, as well as the two lions from the Tehran Zoological Garden due to the consumption of contaminated donkey's meat with *B. mallei* are alarming for the occurrence and rising prevalence of disease in Iran (7).

Next-generation sequencing (NGS) technology has enabled noteworthy advances in molecular typing and epidemiological studies of microorganisms. However, many laboratories not equipped with sequencing tools. Pulsed-field gel electrophoresis (PFGE) is one of the current laboratories 'gold standards' and a powerful DNA-based molecular typing technique applied to distinguish and differentiate genetic relationships in pathogenic isolates of *B. mallei* (7). The prevention, control, and treatment strategies of the glanders are prerequisites to identify and type the *B. mallei* isolates circulating in the country and addressing in our study. The present study aimed to analyze the genomic pattern of *B. mallei* Iranian field isolates by pulsed-field gel electrophoresis (PFGE) discriminatory typing.

MATERIALS AND METHODS

Bacterial isolates, growth conditions and identification. The Razi Vaccine and Serum Research Institute (RVSRI)-archived isolates of *Burkholderia mal-*

lei were addressed in the study (Table 1). In addition to the four wild-type isolates, the standard strain of *B. mallei* Razi 325 (RTCC 2375) was also used. *B. mallei* Razi 325 is the non-native, smooth, and virulent laboratory strain that has been gifted to the RVSRI by Sweden. This strain has been used in the production of the purified protein derivative (PPD)-mallein in the RVSRI since 1956. The RVSRI-archived isolates of *B. mallei* are cultured in tryptic soy broth (TSB) containing 20% glycerol and kept as stock of freeze-dried (-80°C) cultures for the next reviving.

B. mallei isolates from frozen stocks were aerobically cultured in 100 ml nutrient broth supplemented with glycerol 4%, polymyxin E (colistin) 1000 U, bacitracin 250 U, cycloheximide (actidione) 0.25 mg, and horse serum 10% for 48 h at 37°C to get optimal growth. API 20NE identification system (bioMérieux®, France) was used for the biochemical characterization of the bacterial isolates. *B. mallei* isolates were inoculated into sodium chloride solution 0.85%, and turbidity was adjusted to 0.5 McFarland standard. The inoculums were distributed into test cupules and incubated for 24 h at 37°C. Biochemical reactions were read as positive/negative, translated into numerical profiles, and interpreted by APILAB Plus software, version 3.3.3. Further biochemical tests, including catalase, oxidase, Triple Sugar Iron (TSI), Sulfur Indole Motility (SIM), Oxidation-Fermentation (OF), gelatin liquefaction, and nitrate reduction were also carried out on *B. mallei* isolates. All the procedures were performed in a biosafety level 3 (BSL-3).

Inactivation of bacterial isolates. Prior to DNA preparation and PCR assay, the bacterial isolates were heat-inactivated from pure cultures according to the guidelines and precautions for the diagnosis of *B. mallei* infectious agent (8). The bacterial isolates were inactivated at 56°C for 1 hour in a water bath. The viability testing of isolates was checked on blood agar following the incubation at 37°C for 48 h.

Genomic DNA extraction and PCR detection. Genomic DNA of bacterial isolates was extracted using an optimized protocol as described by the World Organization for Animal Health (OIE) (8). In brief, the bacterial colonies were inoculated to the lysis buffer (6 mM Tris-HCl, 1 M NaCl, 0.1 M EDTA, 0.5% SDS) containing 0.5% Tween 20 and 2 mg/ml Proteinase K, and then heated at 65°C for 10 min, fol-

Table 1. The RVSRI-archived isolates of *Burkholderia mallei* used in this study

Isolate	Host	Date of isolation	Location
Tiger	Siberian/Amur tiger	2010	Eram Park Zoo, Tehran, Iran
Kordan	Mare	2013	Kordan, Alborz, Iran
Oshnavieh	Mule	2014	Oshnavieh, West Azerbaijan, Iran
Semirom	Mare	2016	Semirom, Isfahan, Iran

lowed by centrifugation at $16,000 \times g$ for 10 min. The supernatant was purified by a mixture of phenol-chloroform-isoamyl alcohol (25:24:1). Genomic DNA was precipitated using cold isopropanol, then washed with 70% ethanol, and finally dissolved in TE buffer. The quantification and qualification of DNA samples were assayed by NanoDrop™ 3300 Fluorospectrometer (Thermo Scientific, USA).

Molecular identification of bacterial isolates was done based on amplification of *BimA*, IS407-flip, and 23S rRNA target genes (Table 2).

PCR amplifications were carried out using a Mastercycler PeQ STAR Gradient (PeQLab, Germany) in a total volume of 16 μ l mixture containing 8 μ l the ready-to-use $2 \times$ Taq DNA Polymerase Master Mix RED (Ampliqon, Denmark), 0.4 μ l each of primers (5 pmol/ μ l), and 1.5 μ l DNA template (~25 ng). The PCR conditions began with an initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s (for *BimA*) and annealing at 65°C for 30 s (for IS407-flip), and extension at 72°C for 60 s. Then final extension was done at 72°C for 7 min. The 5.0 μ l PCR product obtained was analyzed on 1.5% agarose gel in 0.5x TBE buffer stained with RedSafe™ Nucleic Acid Staining as a visualizing agent and run for 45 min at 90 V.

Pulsed-field gel electrophoresis (PFGE). *B. mallei* isolates were grown in nutrient agar with 1% glycerol, then a loopful of bacterial culture was resuspended into cell suspension solution, CSB, (75 mM NaCl, 25 mM EDTA, pH 8) and harvested at OD₆₀₀

of 1.2 corresponding to a cell density of $\sim 10^9$ cells/ml. The suspension of bacteria was inactivated at 65°C.

The CSB was mixed 1:1 with molten 2% low-melting point ultrapure agarose (Clever Scientific, UK) and pipetted into plug casts. The plugs were placed in cell-lysis buffer, CLB, (50 mM EDTA, 50 mM Tris, 0.1% SDS, and 1% N-lauryl sarcosine sodium (W/V), 500 μ g/ml proteinase K, pH 8) for 16 hours to eliminate the cell components. The plugs were washed three times with Tris-EDTA (TE 1x) buffer, pH 8.0, followed by incubation at 37°C for 15 min. The plugs were cut into 1 \times 4 \times 4 mm fragments and digested by *AfIII* and *VspI* (*AseI*) enzymes (Thermo Scientific, USA) at 37°C for 16 hours before being loaded onto a 1% agarose gel in 0.5x TBE buffer (40 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.3). The wells were overlaid with 1% low melting agarose. PFGE lambda (λ) ladder, 48.5-1000 kb in 48.5 kb increments, concatemers of λ cl857Sam7 lambda DNA was loaded as standard. The PFGE was run on a CHEF MAPPER system (Bio-Rad Laboratories, Hercules, CA, USA) for 18 hours at 6 V/cm, and 14°C, at an included angle of 120° using the following parameters: initial switch time, final switch time, and run time; block 1: 10-50 V for 7 hours, block 2: 30-40 V for 7 hours, and block 3: 50-90 V for 4 hours.

Staining the gel and PFGE pattern analysis. The gel was detached from the cell, transferred into a 0.5 μ g/ml ethidium bromide solution in dH₂O, and stained for 20-30 minutes. The gel was destained in distilled water for 2 hours. DNAs were visualized by

Table 2. The specific primers for PCR amplification of *B. mallei* target genes

Target	Primer sequence (5'→3')	Amplicon size (bp)	Annealing (°C)	Reference
<i>BimA</i>	F: TTCGATCGATTCTGCTATC R: GCGTTAAACGCCGTACTIONTTC	250	56	(9)
IS407-flip	F: TCAGGTTTGTATGTCGCTCGG R: CTAGGTGAAGCTCTGCGCGAG	989	65	(10)
23S rRNA	F: AAACCGACACAGGTGG R: CACCGAACTAGCA	526	58	(11)

placing the gel on a UV transilluminator (254-360 nm). The pattern analysis and dendrogram construction were carried out with BioNumerics version 7.5 (Applied Maths NV, Sint-Martens-Latem, Belgium).

Histopathological analysis. The histopathological analysis of *B. mallei* strains was studied in guinea pig as an animal model. A male guinea pig, with a weight of about 370 g and 9 weeks of age, was administered with a 10^7 CFU/ml crude suspension of heat-inactivated *B. mallei* by 1 ml of the inoculum in the left lower abdominal quadrant intraperitoneally (IP) using a 25-gauge needle. A control group (negative control) of the guinea pig was also injected with 1 ml phosphate-buffered saline (0.01 M PBS, pH 7.2). The guinea pigs were kept in a separate clean cage of an isolator under aseptic conditions and monitored once daily for 72 hours. Bacterial colonization in the testicle and its swelling (also called Strauss phenomenon) was followed after 72 hours. The testicular tissue of the guinea pig was collected and fixed in 10% neutral-buffered formaldehyde, embedded in paraffin wax, processed routinely, and sectioned into 5 μ m thick slices. The tissue sections were then placed on a slide glass and stained with hematoxylin and eosin (H&E) finally, they were examined by light microscopy at $\times 40$ magnification.

RESULTS

Bacterial culture, identification and biotyping characteristics. The small, smooth, and grey colonies of *B. mallei* strains were seen on nutrient agar after 48 h incubation at 37°C (Fig. 1). Catalase positive, oxidase negative, indole negative, H_2S negative, non-motile, no change on TSI, nitrate reduction positive, and growth ability at 37°C were also confirmed for *B. mallei* isolates. The other biochemical profile of isolates was positively found for NO_3^- (potassium nitrate), ADH (L-arginine), GLU (D-glucose), NAG (N-acetyl-glucosamine), GNT (potassium gluconate) by using API 20NE system (Fig. 2). Amplification of IS407-flip and *BimA* genes of *B. mallei* isolates showed the expected sizes of 989 bp and 250 bp, respectively (Fig. 3). PCR amplification of 23 rRNA of *B. mallei* isolates also showed the amplicon size of 526 bp (Fig. 4).

DNA restriction patterns of *B. mallei* strains were successfully generated by PFGE (Fig. 5). The repro-



Fig. 1. The small, smooth, and grey colonies of *B. mallei* on nutrient agar after 48 h incubation at 37°C.

ducibility of PFGE was checked twice by preparing agarose-embedded DNA from 5 isolates of *B. mallei* (Fig. 6). Similarity coefficients between two replicates ranged between 93.5 and 95.2% (mean \pm standard deviation, $94.23\% \pm 1.29\%$). The PFGE types were characterized based on DNA banding patterns. According to both enzymatic digestion patterns by using *AfIII* and *VspI*, we found three different clonal types or clusters, including PFGE type of *B. mallei* Razi 325 strain (Clone I), PFGE type of Tiger, Kordan, and Oshnavieh strains (Clone II), and PFGE type of Semirom strain (Clone III). *B. mallei* Razi 325 was categorized as an unrelated strain which was belonged to different cluster differing by more than four bands. Tiger, Kordan, and Oshnavieh strains were regarded as genotypically indistinguishable being identical patterns. Interestingly, Semirom strain indicated a single band differentiation in digestion patterns of both *AfIII* and *VspI* for PFGE typing, and considered as a related strain.

Histopathological findings. The swollen scrotum, edematous, and orchitis were observed in the guinea pig after 72 h IP injection. The guinea pig was euthanized with chloroform, dissected with the routine techniques, and testicle tissue was immersed in formaldehyde (Fig. 7). The histological evaluations exhibited tubular hyperemia, infiltration of the epididymis, and pyogranulomatous inflammation re-



Fig. 2. API 20NE identification system and biochemical characterization of *B. mallei* isolates

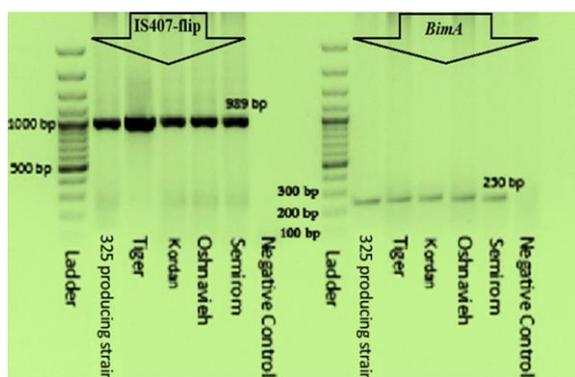


Fig. 3. PCR amplification of IS407-flip (989 bp) and *BimA* (250 bp) genes of *B. mallei* strains.

action in the parenchyma and scrotal areas (Fig. 8). Polymorphonuclear (PMN) leukocytes infiltration was also seen in the testicle tissue which was inconsistently progressed into discrete pyogranulomas.

DISCUSSION

In the current paper, we applied PFGE as a more easily understood and accessible method for high-resolution typing of *B. mallei* isolates. Glanders, the OIE notifiable disease, is recognized as a priority disease for governments in the affected areas considering the zoonotic potential, lack of vaccine availability, easy transmission, and its implications for vulnerability among different populations of the solipeds. The reported cases of *B. mallei* in animals and other accidental hosts such as human are still rare in Iran. However, the implementation of government-sponsored programs is considered a legal obligation for controlling the disease and reducing its losses due to the importance of the *B. mallei*, as a deadly biological pathogen, from the perspective of the Convention on the Prohibition of the Development, Production,

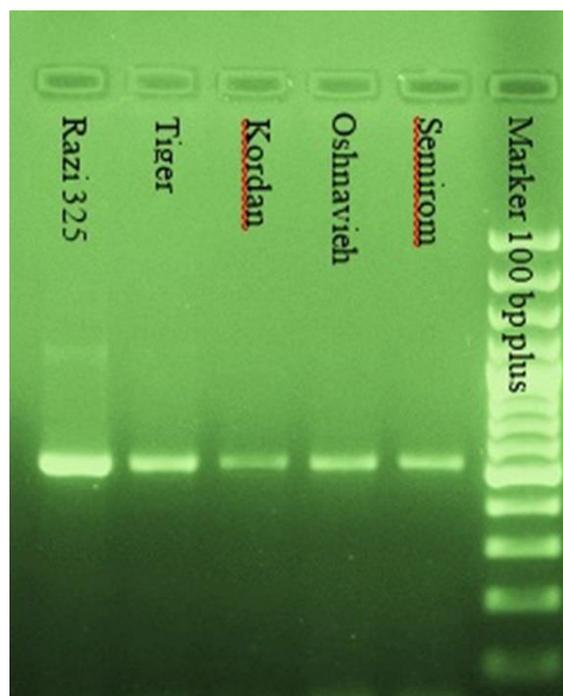


Fig. 4. PCR amplification of 23 rRNA of *B. mallei* isolates (526 bp)

and Stockpiling of Bacteriological (Biological) and Toxin Weapons and on their Destruction (known as the Biological and Toxin Weapons Convention, or BTWC).

Our pathological findings showed that the main gross lesion was orchitis which was observed in the guinea pig after 72 h IP injection of the standard strain of *B. mallei* Razi 325. The main histopathological lesions were pyogranulomatous inflammatory cell infiltrate in the testicular tissue. Currently, not enough data exist on histopathological changes of *B. mallei* in the guinea pig as a susceptible animal model.

Several methods have been used to establish relatedness between *Burkholderia* isolates, including biotyping, serotyping, phenotyping, and genotyping, es-

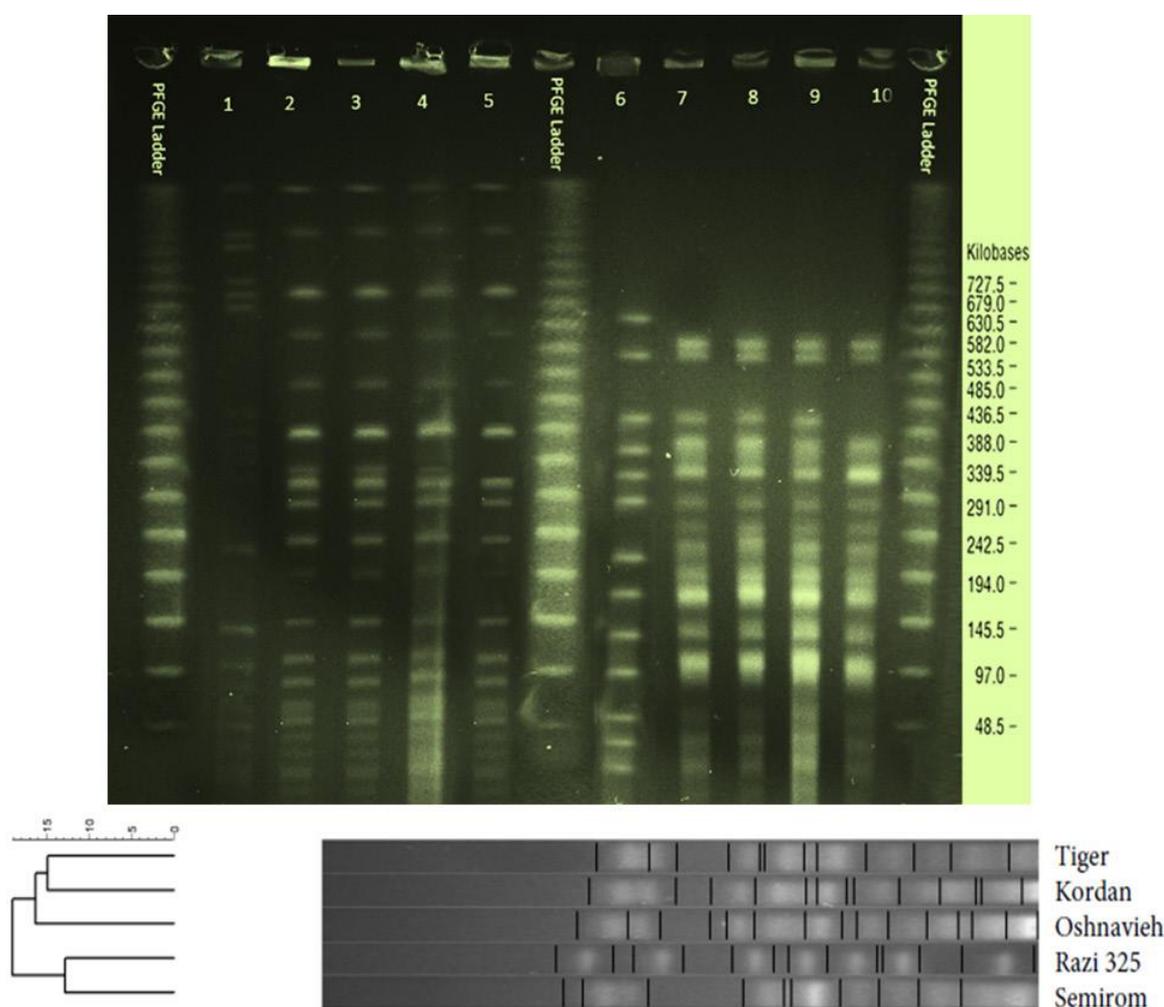


Fig. 5. PFGE algorithm for *B. mallei* strains typing. PFGE-standard lambda (λ) ladder (Promega, USA) in the lateral and central lanes of the gel: 48.5-1,000 kb in 48.5 kb increments (λ cl857Sam7 concatemers); Lanes 1-5: restriction profile by *AfIII*, Lanes 6-10: restriction profile by *VspI*. Lanes 1,6: *B. mallei* Razi 325 strain (Clone I); Lanes 2,7: *B. mallei* Tiger strain (Clone II); Lanes 3,8: *B. mallei* Kordan strain (Clone II); Lanes 4,9: *B. mallei* Oshnavieh strain (Clone II); Lanes 5,10: *B. mallei* Semirom strain (Clone III)

pecially PCR-based fingerprinting techniques. OIE is developing reliable diagnostic protocols for glanders, suggesting PCR-based molecular technique which has been progressed in many OIE-supervised reference laboratories. PCR-based methodologies have been used to detect and differentiate the *B. pseudomallei* complex (*B. pseudomallei*, *B. mallei*, and *B. thailandensis*) as previously described by Lowe et al. (12). The other approaches such as multiplex PCR(13), loop-mediated isothermal amplification (LAMP) assay (14), and TaqMan qPCR/RT-qPCR assay by targeting an SNP in some virulence genes (15) with high accuracy were developed to detect *B. mallei* in recent studies.

PCR study targeting the *Burkholderia* intracellular motility A (*BimA*) gene and the flagella biosynthesis protein insertion element (IS407-flip) has been reported to detect *B. mallei* with an accuracy of 100% (10, 16). *BimA* is responsible for actin-based motility and cell-to-cell spread of *B. mallei* (16). *BimA* is also present in *B. pseudomallei*, although the terminal 300 bp of the gene is over 99% similar to the 3'-end of *B. mallei BimA*, its 5'-end displayed no nucleotide sequence similarities to the same region of *BimA* (17). The insertion element, IS407, has a potential role in the genetic rearrangement of *B. mallei*. The genetic difference of *fliP* (flagellin P) between *B. pseudomallei* and *B. mallei* is used to design specific primers for

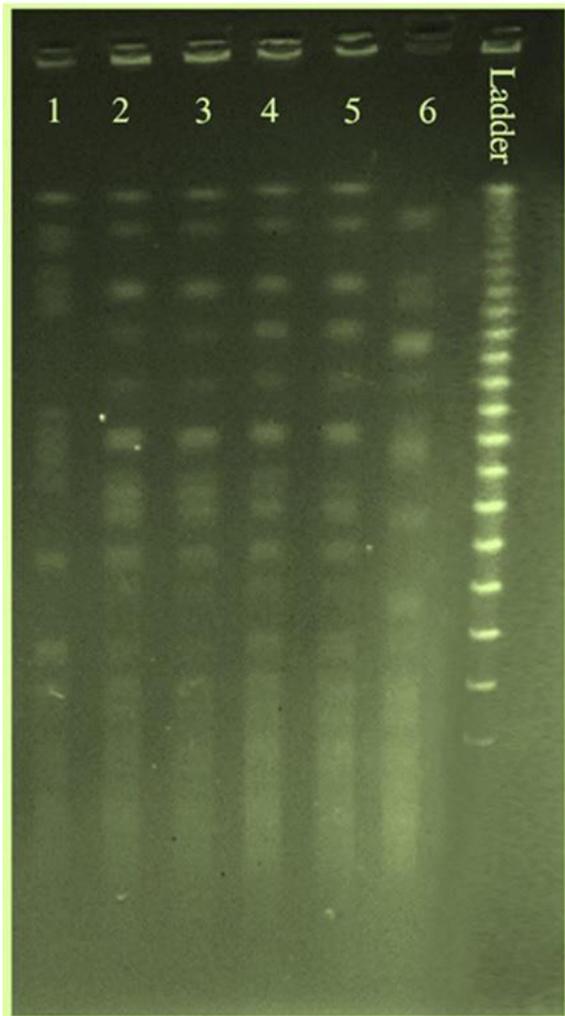


Fig. 6. The reproducibility power test of PFGE. The *AfIII*-digested genomic DNA patterns of *B. mallei* and *B. pseudomallei*. Lanes 1: *B. mallei* Razi 325 strain; Lanes 2: *B. mallei* Tiger strain; Lanes 3: *B. mallei* Kordan strain; Lanes 4: *B. mallei* Oshnavieh strain; Lanes 5: *B. mallei* Semirom strain; Lane 6: *B. pseudomallei* (Razi Type Culture Collection (RTCC)).

the detection of *B. mallei* by PCR (10).

There is little information about the epidemiology and genetic relatedness of *B. mallei* in Iran. PFGE is generally considered the “gold standard” in the molecular typing of microorganisms. Nevertheless, there are only a few studies that have applied PFGE in assessing *B. mallei* epidemiology (18). The genomic approaches using multi-locus sequence typing (MLST) and array-based comparative genomic hybridization (aCGH) clusters have earlier been applied for phylogenetic studies and the relationship of *B. mallei* strains (19). The establishment of interna-



Fig. 7. The Strauss phenomenon and testicle swelling of guinea pig following the intraperitoneal (IP) injection of *Burkholderia mallei*

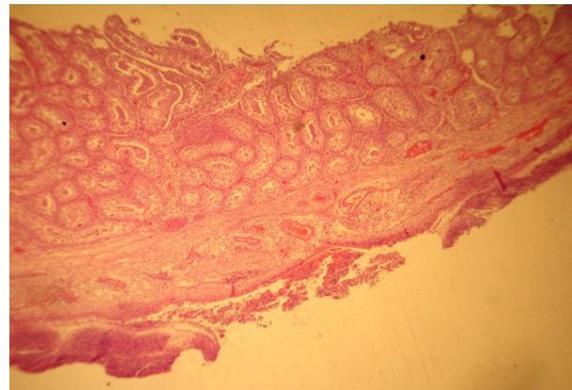


Fig. 8. The cross section of testicle tissue of male guinea pig 72 h after intraperitoneal (IP) injection of *B. mallei* (Hematoxylin and Eosin, H&E, staining $\times 40$): The tubular hyperemia, infiltration of the epididymis, and pyogranulomatous inflammation reaction in the parenchyma and scrotal areas.

tional outbreak detection networks and standardization of the approaches for pathogen phylogeny and population studies have been provided by contemporary typing methods such as PFGE and MLST (20). PFGE includes surrounding organism in agarose, in situ lysing organism, and digesting genomic DNA with restriction endonucleases. Genomic DNA fragments embedded in agarose plugs are inserted into gel wells, resolved into the pattern of separate bands on agarose gel, then compared with one another to determine their relatedness.

The PFGE types were defined according to the band pattern similarities. The closely related isolates were detected based on one to three band differences, while identical isolates showed no band differences.

PFGE typing of *B. pseudomallei* by using the restriction endonucleases *Xba*I and *Spe*I has previously been described (21, 22). A study showed that the discriminatory power of *Spe*I was higher than the *Xba*I for PFGE typing of clinical *B. pseudomallei* isolates (22). This finding may be generalized for PFGE typing of *B. mallei* since *B. mallei* is believed to have originated from *B. pseudomallei*. However, it seems that *B. mallei* contains numerous IS elements resulting in multiple deletions and rearrangements of the genome relative to *B. pseudomallei* (23). A good resolution by PFGE using *Spe*I together with clustering of some geographically related *B. mallei* isolates was demonstrated by Chantratita et al. (24). Selecting suitable restriction enzymes is significant to distinguish *B. mallei* strains with high resolution in PFGE. We successfully applied *Af*III and *Vsp*I with high resolving power in PFGE typing of *B. mallei* within the shortest running time (18 h) showing three different clone types of *B. mallei* isolates. The reproducibility of both enzymes was also checked and deduced the same remarkable results. This is the first report on PFGE analysis of *B. mallei* by the newly established enzymes *Af*III and *Vsp*I.

In conclusion, we suggest that PFGE is feasible for reference laboratories that will be confronted primarily with outbreaks of glanders. Developing a database for PFGE banding pattern analysis of *B. mallei* may be a valuable resource in the future outbreak of animal or human disease.

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