Use of PCR for detection of *Burkholderia mallei* in Türkiye

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ABSTRACT

Within the scope of the "National Ruam Eradication Project" carried out between 2000-2001, Glanders was eradicated in our country. Unfortunately, 81 horses were culled in Türkiye in December 2019, following the detection of Epidemic in horses in Büyükada. In 2019, Glanders were reported in horses in Uşak and Bolu. No human cases have been reported. Türkiye is at risk for Glanders because of its geography. Therefore, reliable and fastly detection of *Burkholderia mallei* by PCR in a short time will prevent the distribution of unwanted infections that may occur in the future throughout the country. In this study; *Burkholderia mallei* strains isolated from horses were verified and optimized by PCR. The use of PCR for the detection of *Burkholderia mallei* was performed for the first time in our country. It has been concluded that the PCR as a diagnostic method with high reliability and sensitivity safely used together with diagnosis of Glanders.

Chemical Biological, Radiological Nuclear Threats (CBRN) is on the agenda of world health today as they can cause mass deaths by being used as weapons. One of the bacteria that has the potential to be used in biological attacks is *Burkholderia mallei* (*B. mallei)*. It is included in the Bioterrorism Factors and Diseases List (Category B) made by the United States Center for Disease Protection and Control, the List of Important Dangerous Factors of the Biological Weapons Convention and the List of the European Union Bioterrorism Working Group. *B. mallei* is the causative agent of Glanders. Glanders is a systemic and zoonotic infection of equine animals with contagious character, acute and chronic course (20). Glanders is characterized by the formation of purulent nodules and ulcers in the skin, respiratory, and internal organs of patients. The disease can often be transmitted by direct contact, or it can also be transmitted aerogenously. Contamination of the food and water sources is generally seen as a result of the contamination of the environment with the nasal secretions of infected animals (1, 2). The disease follows an acute and chronic course. While it has an acute course in donkeys and mules, it has a chronic form in horses. In humans, in acute events; it progresses with 95% mortality and death occurs within 3 weeks. Treatment is possible with the use of antibiotics (2, 15).

The first information about the presence of Glanders in Türkiye was found in letters written by Veterinarian Godlewsky (9, 19). In these letters, it is mentioned that animals with Glanders are used as long as they can work and then they were released when they were unable to work. This led to the rapid spread of the disease. Glanders, which caused significant damage to the national economy, human and animal health, spread rapidly during the Balkan, I. World and War of Independence showed an epidemic course. As a result of this epidemic, it has been reported that animals infected with the disease are used in the army without health checks (18). In the Public Health Law enacted in 1930 in Türkiye, it is mentioned that the notification of Glanders, the isolation of the sick animals, the transfer of the deceased animals, and the keeping of

culture of the bacteria in the laboratory are prohibited. Until 1999, cases were reported from various places in our country. Within the scope of the "National Ruam Eradication Project" carried out between 2000-2001, the Equidae animals available in our country were tested and the carrier animals were destroyed (25). Isolated cases of Glanders in horses are also reported from time to time in Türkiye.

The aim of the study was to optimize the PCR detection of *B. mallei* against possible infection with Glanders. In this study, 14 isolates isolated between 1985 and 2000 in Veterinary Control Central Research Institute and NH strain were used. The isolates from enriched Dorset-Henley medium were purified in a biosafety level-3 laboratory. For the detection of the *B. mallei*, manufacturer's instructions for High Pure PCR Template Preparation Kit (Roche Diagnostics, Germany) were followed in order to isolate DNA. Primers used for the detection of *B. mallei* are shown in Table 1.

Following that, the primers targeting the 823 bp gene of *B. mallei* used by Merwyn et al. (13) were used in PCR. DNAs were stored at -20 °C. The DNA was stored at -20 °C until analysis.

In this study, the NH strain used in mallein production in our institute was used as a positive control. An optimization study was performed with NH strain used in mallein production in Veterinary Control Central Research Institute to determine the optimal working concentration of synthesized primers. Dilutions $3x10^{1}$ to $3x10^{8}$ of NH strain were prepared. 1 ml of these dilutions were absorbed into the swabs and genomic DNA extracted from each dilution was tested in 5 replicates. Then, PCR process was performed. PCR yielded successful results in all dilutions. This shows us that although there are very few pathogens, the PCR process reliably detects the causative agent. In this way, the disease will be detected in the early period when the clinical symptoms of the disease are not seen and the spread of the disease will be prevented.

According to the results of this study, 50 ng template DNA, 1.0 µM of each primer pairs (BM-4 ve BM-5), 1.25 U of Taq DNA polymerase, 200 µM dNTP, 1.5 mM MgCl, DNase & RNase free water and 1x PCR buffer were arranged in 25 µl reactions. Class-2 laminar cabinet was used for mixing reagents, other buffer liquids and PCR and Isolation. The heat cycle was adjusted as 95°C pre-denaturation for 5 min, 35 cycles at 95°C for 1 min for denaturation, and 60.6 °C for 2 min and 72°C for 2 min. This was followed by 1 cycle at 72°C for 10 min. The reaction mixture was subjected to electrophoresis on 1.2% agarose gel to analyze (Figure 1).

In our study, we performed the DNA extraction and PCR analysis of 14 different samples which were isolated between 1985 and 2000 as *B.mallei* which is a zoonotic and notifiable bacterial dissease causing high mortality in equidae. After the analysis of the results, it is concluded that PCR is a verified method for the detection of *B. mallei*.

Table 1. Primers used in study to amplify the Glander Disease and Amplicon size.

BM-4 5'-CGA TCC TGG TGT GCT CGG CCG_3'	823 bp
BM-5 5'-CGC AGA CCT TCT TCC ATC GCG ATC-3'	823 bp

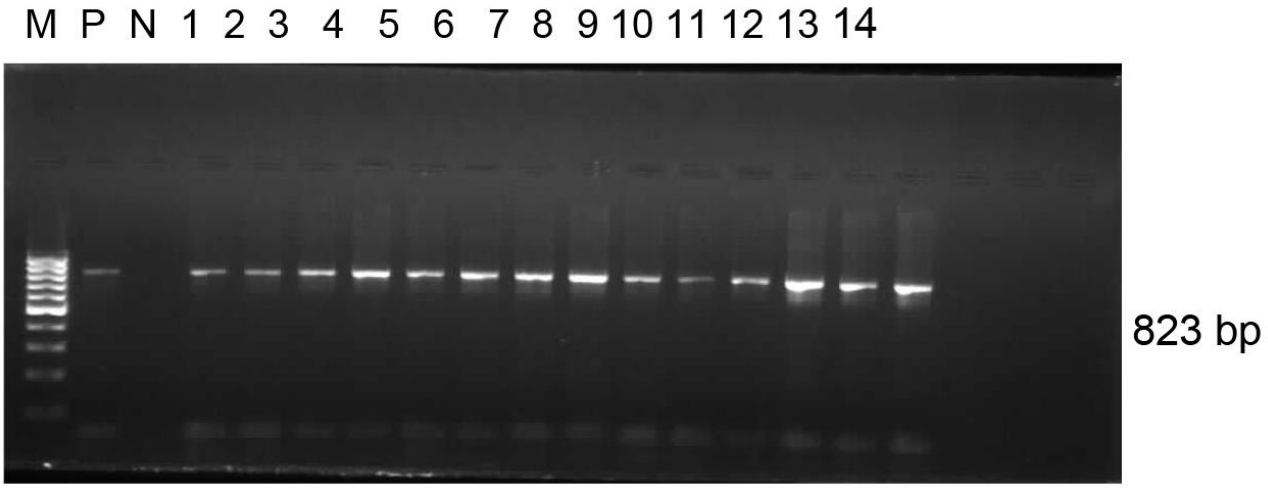

Figure 1. PCR for B mallei strains. Lane M: 100bp ladder; Lane P: Positive control; Lane N: Negative control; Lane 1-14: *B. mallei* isolates.

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Many methods are used in the diagnosis of Glander Disease. Mallein and serological tests are the most important tests in the diagnosis of the disease. Mallein test is a specific and high sensitive test. During the application of this test, intradermo-palpebral or intradermo-cervical injections cause a reaction characterized with swelling of the skin is observed within 1-3 days in infected animals. Intradermic Sauton Mallein produced in our institute is a biological test material accepted and recommended by the OIE (16). Serological tests are Complement Fixation Test (CFT) and Enzyme-Linked Immunosorbent test (ELISA). CFT which has a good sensitivity as a screening test and is able to detect chronically infected glanders carriers. And CFT is prescribed by the World Organisation for Animal Health (OIE) for international trade of equines (17). This method remains difficult to standardise. CFT reliability depends on the choice of antigen and protocol, hence, specificity and sensitivity of the CFT test may vary and yet the sensitivity of this test may range from 62.5% to 100%. In addition to those, the Rose-Bengal plate test is applied in some countries. Specific monoclonal antibodies, molecular methods, western blotting are among the other diagnostic methods used. In the early stages of the Glanders when clinical signs are mild, anamnesis and laboratory diagnoses are not sufficient for the diagnosis of the disease. Therefore, the use of methods such as PCR that are able to detect small numbers of pathogen for the diagnosis of *B mallei* is very important in terms of preventing possible epidemics (3, 6-8, 10, 11, 13, 14, 21, 22).

Molecular methods are used to support and confirm the diagnosis. It is used as a fast, sensitive and reliable diagnostic tool both in diagnosis and verification. Gee et al. (5) used 16S rRNA gene sequencing for rapid identification and differentiation *of Burkholderia pseudomallei* and *B. mallei*. Scholtz et al. (24) used the 989 base pair gene regions of the bacterial flagellar antigen in the Glander Disease epidemic in the United Arab Emirates and the diagnosis of *B. mallei* was made that way. Merwyn et al. (13) successfully optimized *B. mallei* inoculated environmental samples with the primers they designed and detected Glander Disease quickly and reliably. Today, with the rapid advancement of technology, whole genome sequencing of *B. mallei* has been done. Thus, the epidemiology of the disease is tried to be understood. Fonseca et al. (4) identified 2 new genotypes of B. mallei in Brazil by Whole Genome Sequence (WGS). These study results show different introduction events regarding glanders in Brazil, including strains of European origin, in connection with colonization or trade. Singha et al. (23) identified targeting *B. mallei* strains in India and Pakistan by whole genome sequence of 10 *B. mallei* strains with four new markers. In this study, new SNP markers were determined as a result of SNP analysis. Such rapid and distinctive typing tools will contribute to the epidemiological monitoring of *B. mallei* infections in endemic regions of the disease. WGS could help in elucidating the origin of the disease.

Glanders is listed as one of the notifiable diseases by the World Organization for Animal Health (WHO) due to the debilitating disease it causes in both animals and humans. The eradication programs applied against glanders disease, which spreads widely all over the world and causes great losses in equidae, started to give results especially in the first half of the 20th century (17). Today, some outbreaks of Glanders have been reported in Afghanistan, Bangladesh, India, Pakistan (South Asia), Bahrain, Iraq, Syria, Iran, Kuwait and United Arab Emirates (The Middle East), and Brazil (South America) (12). In the last 25 years, a marked increase in outbreaks or cases leading to a putative relapse has been observed (17, 18). It is worth considering that the disease is probably often misdiagnosed and certainly underreported in many countries (18).

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Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

ES, OD, DD and DA conceived and planned the experiments. ES and OD carried out the experiments. ES, OD and DD planned and carried out the simulations. ES, OD, DD and DA contributed to the interpretation of the results. ES took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement

This study does not present any ethical concerns.

Animal Welfare

Not applicable.

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