Molecular Epidemiology of Glanders, Pakistan

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Glanders is an equine disease that was recognized by Hippocrates and Aristotle (1). It is caused by the bacterium Burkholderia mallei, an obligate pathogen of horses, donkeys, and mules (Equidae), with occasional infections in felines, canines, and humans (2,3). Strict regulation of equines has reduced the range of this once globally distributed disease to a few endemic foci in South and Central America, the Middle East, and parts of Africa and Asia (2,4). This emerging disease has only recently regained attention following the listing of B. mallei as a Category B agent by the US Centers for Disease Control and Prevention (2). Although outbreaks are common in regions of disease endemicity, much of what is known about the ecology and natural population dynamics of B. mallei relies on indirect evidence and expert opinion, with little to no knowledge concerning its genetic diversity (2,3). We genetically characterized 15 samples of B. mallei from recent outbreaks in Pakistan to provide additional knowledge of how this disease of antiquity is transmitted throughout endemic regions today.

The Study

We obtained clinical samples and background information from 15 glanderous equids in Punjab, Pakistan from 1999 through 2007 (Table; online Appendix Table, available from www.cdc.gov/EID/content/15/12/2036-appT.htm). Research on equine subjects was approved by the Synopsis Scrutiny Committee and Animal Ethics Committee, Faculty of Veterinary Science, University of Agriculture, Faisalabad, Pakistan. Samples were plated on brain-heart infusion (BHI) agar with 4% glycerol and incubated for 24–30 hours at 37°C. Individual colonies were inoculated into BHI broth containing 4% glycerol and were incubated with shaking for 36 h at 37°C. An aliquot of broth (1.5 mL) was centrifuged at 13,000 rpm for 15 min. Genomic DNA was extracted from the resulting pellets using standard digestion buffer and phenol-chloroform extraction protocols (6).

For genotyping, we screened 23 loci (online Appendix Table) from a previously established 32-marker multiple locus variable number of tandem repeats (VNTR) analysis system designed for B. pseudomallei and B. mallei (7). In silico genotyping of the same loci was also performed for 10 whole genome sequences (WGS) of B. mallei (8; online Appendix Table). VNTR markers have higher mutation rates than other genetic markers, which makes them inappropriate for determining deep levels of evolutionary relatedness. VNTRs, however, are appropriate for 1) discrimination between closely related isolates, 2) determination of the degree of relatedness among isolates, and 3) discernment of population structure on a spatial scale (7,9,10). This utility is especially important for B. mallei because it is a recently emerged clone of B. pseudomallei and has been shown to be genetically monomorphic with typing methods such as multilocus sequence typing (11). To compare the genetic diversity of our Punjab isolates to that of sequenced strains, we performed a phylogenetic analysis on the 23 loci using the neighbor-joining algorithm in PAUP* 4.0b (9). To determine the genetic relationships among the Punjab population itself, we performed the same analysis using only the Punjab isolates and polymorphic loci (n = 15 loci).

Combined analysis of the Punjab isolates and WGS showed that the Punjab isolates are phylogenetically distinct from WGS (Figure 1). This finding was also demonstrated in the values for average pairwise distance (APD), where the APD among Punjab isolates is 2× lower than the APD calculated for either the entire phylogeny or the WGS alone (Figure 1). Therefore, the Punjab isolates represent only a small amount of the genetic diversity demonstrated in this pathogen. Phylogenetic analysis of the Punjab isolates alone placed 14 of the 15 samples into 3 distinct clades with 1 sample standing alone (Figure 2). Most samples (9/15) belong to clade A, whose isolates are both
temporally and geographically diverse, suggesting that this lineage is ecologically established in Punjab.

Because of the limited sample size, many of the patterns observed from these data may result from sampling bias. However, even a limited amount of genotypic data can be useful in formulating hypotheses regarding the dispersal of *B. mallei*. For example, the presence of samples from Faisalabad in each clade suggests that this district may be a center of diversity in the province (Figure 2) but this does not indicate a lesser degree of diversity in other districts where fewer samples were collected.

The diversity seen in the district of Faisalabad may result from either 1) the industrial nature of Faisalabad or 2) from high endemism of *B. mallei* in the region. Currently, the district has ≈10,000 horses and mules and >44,000 donkeys, plus other transient equines (12). Many equines move through and work in the city, potentially introducing strains from surrounding regions. Because horses and mules can be positive but asymptomatic for glanders (13), many hosts are available to maintain strains throughout the region. Communal stables and water troughs are common throughout the district and *B. mallei* has been isolated from these water troughs (A. Naureen, unpub. data). Furthermore, *B. mallei* can remain viable in contaminated stables for up to 6 weeks (14) and in sterile tap water for up to 4 weeks (15), which provides an environment for establishment and retention of *B. mallei* populations in Faisalabad.

Combining phylogenetic with epidemiologic data reveals how *B. mallei* disseminates throughout a region. For example, epidemiologic data suggests that 2 horses from a farm in the district of Sargodha (PRL3 and PRL4) contracted glanders while at a polo club in the Lahore district. This is supported by VNTR data, as these 2 isolates clustered phylogenetically with one of the samples obtained from an outbreak that occurred at the same polo club 3 months prior (groups 5 and 6, Table). Furthermore, at the time of the PRL3 and PRL4 infections, a co-resident mule with no previous travel history (PRL44) was negative for glanders, making it unlikely that these horses acquired glanders from their farm. This mule was positive for glanders ≈1.5 years later, and the isolate obtained from its infection clustered phylogenetically with the samples from the polo club and

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**Figure 1.** Unrooted neighbor-joining tree based on 23 variable number tandem repeat loci demonstrating that the Punjab isolates (black text and PRL-20) are genetically distinct from and less diverse than available whole genome sequences (WGS, red text) (7). Statistical supports for branches based on 1,000 bootstrap iterations are shown. Sample PRL-20 is shown in red text because it is also available as a whole genome sequence; therefore, it was used in all 3 situations where an average pairwise distance (APD) was calculated. Among 10 WGS, the average pairwise distance was 0.607; between 10 WGS and Punjab isolates, average pairwise distance was 0.627; and among 15 Punjab isolates, average pairwise distance was 0.312. These results indicate that the Punjab isolates are more closely related to each other than to the sequenced strains because the APD among Punjab isolates is 2× lower than the APD calculated in the other 2 situations.

**Figure 2.** Unrooted neighbor-joining tree showing phylogenetic relationships among 15 samples of *Burkholderia mallei* from the Punjab Province, Pakistan. Statistical support for each branch derived from 1,000 bootstrap iterations are shown. Sample names are color-coded to match their district of origin in reference to the inset map of the Punjab Province. Approximate linear distances between districts are Faisalabad to Lahore ≈120 km, Faisalabad to Sargodha ≈84 km, Lahore to Sargodha ≈168 km.
man-mediated movement of equines can in

...transferred a distance of 168 km, demonstrating that hu-

...Nevertheless, this case shows a strain that was

...differed nonsubstantially (data not shown). Therefore, only 3 isolates

...were evaluated by using VNTR. Strains PRL11 and PRL13 were isolated

...from horses that were kept at 2 stables >8 km from each other, but

...the 2 stables had a history of mixing.

...Sargodha horses. Therefore, we hypothesize that the in-

...fectected horses either directly transferred the disease to the

...mule or they contaminated a source on the farm which sub-

...sequently led to the mule’s infection. Environmental sam-

...pling would be required to identify the original infection

...source for the horses and subsequent transmission route to

...the mule. Reports indicate that these animals drank

...from communal water troughs available in different zones of Faisalabad.

...Strains were collected from an outbreak among Faisalabad Mounted

...Police Horses (n = 18) in June 1999. Biochemical test results (based on

...Analytical Profile Index 20E strips; BioMérieux, Marcy l’Etoile, France) differed nonsubstantially (data not shown). Therefore, only 3 isolates were evaluated by using VNTR. Strains PRL11 and PRL13 were isolated from horses that were kept at 2 stables >8 km from each other, but the 2 stables had a history of mixing.

...Sargodha (PRL3, PRL4) participated in matches at the Lahore Polo

...Club. Horses were returned to their farm in late spring 2005. In the fall of

...2005, there was a glanders outbreak at the Lahore Polo Club (see

...Group 5 above). In December 2005, the 2 horses on the Sargodha farm

...tested positive for glanders after being housed together during the

...winter. A mule (PRL44) that was also present at the Sargodha farm

...tested negative for glanders at this time. Approximately 2 years later, the

...same mule tested positive for glanders after reports of 6 months’

...standing nasal discharge. Records indicate the mule was brought to the

...farm at a young age from the city of Multan and never left the farm

...before onset of symptoms.

...Sargodha horses. Therefore, we hypothesize that the in-

...fectected horses either directly transferred the disease to the

...mule or they contaminated a source on the farm which sub-

...sequently led to the mule’s infection. Environmental sam-

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...source for the horses and subsequent transmission route to

...the mule. Nevertheless, this case shows a strain that was

...transferred a distance of 168 km, demonstrating that hu-

...man-mediated movement of equines can influence the dis-

...tribution of *B. mallei* genotypes. This case also suggests that a strain can persist for ≈1.5 years.

...Other cases in the province demonstrate that infections either stem from similar strains or are caused by multiple strains. For example, samples that were placed in the same epidemiologic group cluster together phylogenetically (groups 1, 2, and 4; Table), indicating communal infections similar to the cases described above. In contrast, epidemiologic group 5 (PRL19 and PRL20) was separated into 2 distinct clades (Figure 2), indicating that this outbreak was caused by multiple strains. Therefore, it should not be assumed that an outbreak of glanders is always caused by a single strain.

**Conclusions**

Our study suggests that numerous lineages of *Burkholderia mallei* are present in Punjab, Pakistan, and that these lineages persist across geographic space and time. Despite this, these isolates appear to be genetically dis-

...tinct from other studied strains. The economics and use

...of equines likely contribute to the persistence of glanders

...in this region because modern methods for control of this

...disease (monitoring and euthanasia) are not viable options.
Therefore, other solutions to curbing the spread of glanders need to be identified. We suggest that a focus on finding methods to improve the sanitary conditions of communal water troughs and stables may lead to a practical solution for disease reduction and containment. Finally, our study demonstrates the utility of VNTRs paired with extensive epidemiologic data for analyzing the distribution of \textit{B. mallei} genotypes throughout endemic regions.

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