Virulence of Selected *Mycobacterium tuberculosis* Clinical Isolates in the Rabbit Model of Meningitis Is Dependent on Phenolic Glycolipid Produced by the Bacilli

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Infection with *Mycobacterium tuberculosis* in humans results in active disease in ~10% of immune-competent individuals, with the most-severe clinical manifestations observed when the bacilli infect the central nervous system (CNS). Here, we use a rabbit model of tuberculous meningitis to evaluate the severity of disease caused by the *M. tuberculosis* clinical isolates CDC1551, a highly immunogenic strain, and HN878 or W4, 2 members of the W/Beijing family of strains. Compared with infection with CDC1551, CNS infection with HN878 or W4 resulted in higher bacillary loads in the cerebrospinal fluid and brain, increased dissemination of bacilli to other organs, persistent levels of tumor necrosis factor–α, higher leukocytosis, and more-severe clinical manifestations. This pathogenic process is associated with the production by HN878 of a polyketide synthase–derived phenolic glycolipid (PGL), as demonstrated by reduced virulence in rabbits infected with an HN878 mutant disrupted in the *pks1-15* gene, which is required for PGL synthesis.
pared with that induced by the laboratory strains H37Rv and HN878. Well-organized granulomas with high levels of TNF-α, interleukin (IL)-6, IL-10, IL-12, and interferon (IFN)-γ mRNA were observed sooner in the lungs of mice infected with CDC1551 [11]. Infection with HN878 induced weak T cell proliferation and IFN-γ production by spleen and lymph node cells [13], in association with early death (hypervirulence) in infected mice. In rabbits infected by aerosol with Erdman, H37Rv, or CDC1551, the third strain appeared least virulent, requiring the highest number of inhaled bacilli to form 1 grossly visible pulmonary tuberculosis [15].

Recently, a highly bioactive lipid—a polyketide synthase (PKS)–derived phenolic glycolipid (PGL)—produced by HN878 and W4 but not by CDC1551 or H37Rv was identified and characterized [16, 17]. The PGL-deficient mutant of HN878, constructed by disrupting the pks1-15 gene cluster, was found to be more immunogenic and caused delayed death of the infected mice [16]. Here, we use the rabbit TBM model to study infection with CDC1551, HN878, W4, or H37Rv, and the PGL-deficient mutant HN878pks1-15::hyg is compared with the parental strain.

MATERIALS AND METHODS

Clinical isolates of M. tuberculosis. The M. tuberculosis strains studied were as follows: (1) CDC1551, provided by T. M. Shinnick, Centers for Disease Control and Prevention, Atlanta, GA; (2) HN878, provided by J. M. Musser, Houston, TX [18]; (3) W4, provided by B. N. Kreiswirth, Public Health Research Institute, Newark, NJ; (4) HN878pks1-15::hyg, a mutant strain of HN878 with a pks1-15::hyg, and W4 but not by CDC1551 or H37Rv was identified and characterized [16, 17]. The PGL-deficient mutant of HN878, constructed by disrupting the pks1-15 gene cluster disrupted by insertion of a hygromycin resistance gene, provided by M. B. Reed, Rockville, MD; and (5) H37Rv (TMC no. 102; Trudeau Institute). All procedures were performed in a biosafety level 3 laboratory.

Genotypic differentiation of M. tuberculosis strains: CDC1551 versus HN878 and HN878 versus HN878pks1-15::hyg. To differentiate CDC1551 and HN878, a multiplex polymerase chain reaction (PCR) based on the strain-specific insertions of IS6110 elements in 2 M. tuberculosis chromosomal regions—mmpL4 and oriC—was used. Chromosomal DNA was isolated from single colonies grown on 7H11 agar plates by incubation in Tris-EDTA buffer at 80°C, boiling (5 min), and centrifugation at 15,366 g. Four primers from the GeneAmp PCR System 270 were used under the following conditions: 94°C for 5 min; 35 cycles of 94°C for 20 s, 62°C for 10 s, and 72°C for 15 s; and 72°C for 2 min. The primer pairs used were as follows: E-F (5'-AGCTTCTCTGGCCTCC-3') and E-R (5'-ATCCCGA-TGTTGATAGTC-3'), which amplify a 639-bp fragment in HN878; and A4-R (5'-GTTGATCGCATCACTCTTGAC-3') and dnaA-F (5'-CGCCATCGCCTCAAACGCGC-3'), which amplify a 394-bp fragment in CDC1551. To differentiate HN878 from HN878pks1-15::hyg, a multiplex PCR was performed using the primers pks 5F (5'-ACGTTGATCATGAGCGCCCATCGCA-3'), pks 7R (5'-AGCTTCTCTGGCCTCC-3'), hyg 3F (5'-AGAGCTGCAGAAGCAGCAGAATT-3'), and hyg 1R (5'-CCAGACGCTGTCCAGTCCGGCA-3'). DNA from the mutant strain yields 2 PCR products, of 950 and 580 bp, whereas the parent strain, HN878, yields only the 950-bp product. A total of 573 M. tuberculosis colonies were analyzed (326 for the CDC1551 vs. HN878 comparison and 247 for the HN878 vs. HN878pks1-15::hyg comparison) (see figure A1 in the Appendix, which appears only in the electronic edition of the Journal).

Induction of meningoitis. New Zealand White rabbits (Ganvance Research Products) were used, as described elsewhere [7]. On the day of experiment, rabbits were anesthetized and immobilized. A spinal needle was used to withdraw 0.3 mL of cerebrospinal fluid (CSF) and to inject 0.2 mL (5 × 10^7 CFU) of M. tuberculosis intracisternally. After 2 h, CSF was obtained and plated onto 7H11 agar (Difco), to determine the inoculum. CSF and blood samples were obtained from rabbits weekly for 8 weeks. Half of the brain and part of the lung, liver, and spleen were used for the colony-forming unit assay. The rest of the brain, liver, lung, and spleen were fixed in 10% buffered formalin acetate (vol/vol) (Fisher Chemical) for histopathologic examination. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Medicine and Dentistry of New Jersey, Newark campus, and at the Public Health Research Institute.

CSF samples. CSF samples were analyzed for total leukocyte counts (Coulter Electronics), differential counts (Diff-Quick; Baxter), and colony-forming units. The remaining CSF was centrifuged, and the supernatant was stored at −70°C for the TNF assay.

Blood samples. Heparinized blood, collected from the ear artery, was centrifuged at 10,000 g. Plasma was separated and frozen at −70°C for the TNF assay.

TNF assay. TNF biological activity was evaluated using a cytotoxicity assay for murine L929 fibroblasts, as described elsewhere [7]. This assay does not discriminate between TNFs.

Colony-forming unit assay. Colony-forming units were evaluated in the CSF and organ homogenates by plating 10-fold serial dilutions onto Middlebrook 7H11 agar (Difco).

Clinical scoring system for rabbits. To evaluate the clinical course of CNS infection in rabbits, we developed the following scoring system: stage 0 (normal), stage 1 (hyperesthesia, head tilt, and lethargy), stage 2 (monoparesis), stage 3 (hemiparesis and recumbency), stage 4 (quadriplegia), and stage 5 (anorexia, and CNS depression progressing to moribund state and death). Rabbits at stages 4 and 5 were killed as required by our IACUC.

Histopathologic analysis. Formalin-fixed brains were cut transversely in serial sections from rostral to caudal, representing the fore-, mid-, and hindbrains; embedded in paraffin; sectioned; and stained with hematoxylin-eosin and Ziehl-Neelsen.

Statistical analysis. The independent Student’s t test or the
Bacillary load in cerebrospinal fluid (CSF) and tissues of rabbits infected intrathecally with *M. tuberculosis*. A, No. of *M. tuberculosis* colony-forming units in the CSF of rabbits infected with CDC1551 (white diamonds) \((n = 10)\), HN878 (black triangles) \((n = 12)\), W4 (black circles) \((n = 10)\), or H37Rv (white squares) \((n = 10)\). The clearance of CDC1551 differed significantly from that of other strains \((P = .01)\). B, Colony-forming units in the brains, lungs, and livers 8 weeks after infection of rabbits with CDC1551 (hatched bars), HN878 (black bars), W4 (gray bars), or H37Rv (white bars) (in brain, CDC1551 vs. HN878, \(P = .02\)). No dissemination of CDC1551 to the lungs \((P > .001, \text{ vs. HN878 and W4})\) or livers \((P > .001)\) was seen. Values are means ± SEs.

Mann-Whitney U test for nonparametric independent data was used for analyses. The Kruskal-Wallis test was used to determine statistical differences in the clinical manifestation scores between different strains. \(P < .05\) was considered to be significant.

**RESULTS**

**Bacillary load in CSF and tissues of rabbits infected intrathecally with different *M. tuberculosis* clinical isolates.** Rabbits were infected by intracisternal inoculation of \(5 \times 10^5\) cfu and were monitored for 8 weeks. At 2 h after infection, 4 log_{10} cfu/mL of CSF were detected for all 4 *M. tuberculosis* strains (figure 1A). By 7 days after infection, a reduction in the number of colony-forming units was noted for all strains. Thereafter, complete clearance of CDC1551 from the CSF started earlier (by 2 weeks after infection; \(P = .01\)), and, from 4 weeks after infection, no viable bacilli were detected in the CSF. In contrast, numbers of HN878 and W4 remained elevated for 8 weeks. H37Rv persisted up to 5 weeks and was then fully cleared. At 8 weeks after infection, HN878 showed the highest bacillary load in the brain, compared with the other strains \((P = .02, \text{ vs. CDC1551})\) (figure 1B). No dissemination of CDC1551 to the lungs was noted at day 14 and day 21 after infection (data not shown) or at 8 weeks after infection \((P > .001, \text{ vs. HN878 and W4})\). Similar results were seen in the liver \((P > .001)\). The high loads of HN878 in the lungs suggested local replication and/or continuous dissemination from the CNS.

Inflammatory response and clinical course of disease in rabbits with meningitis. A, Leukocyte density in the cerebrospinal fluid (CSF) of rabbits infected with CDC1551 (white diamonds), HN878 (black triangles), W4 (black circles), or H37Rv (white squares). HN878- and W4-induced leukocyte densities were higher than those induced by CDC1551 at 1 and 2 weeks after infection \((P = .001 \text{ and } P = .01, \text{ respectively})\). Values are means ± SEs. B, Tumor necrosis factor (TNF) level in the CSF means ± SEs of rabbits infected with CDC1551 (hatched bars), HN878 (black bars), W4 (gray bars), or H37Rv (white bars). Values are means ± SEs. C, Severity of signs of disease (clinical score) in rabbits infected with CDC1551 (dark gray area) \((n = 12)\), W4 (light gray area) \((n = 9)\), CDC1551 \((n = 10)\), or H37Rv \((n = 10)\). CDC1551 and H37Rv did not induce neurologic signs. Rabbits infected with HN878 or W4 showed more-severe progressive signs of disease than did those infected with CDC1551 and H37Rv \((P < .0001)\). Results are expressed as the area under the curve.
rabbits with CNS infection. After infection with CDC1551, the leukocyte influx into the CSF was low and dropped slowly (≤1000 cells/mm³) (figure 2A). In contrast, at 1 and 2 weeks after infection, HN878 and W4 leukocyte influx was higher and persisted longer than that of CDC1551 (P = .001 and P = .01, respectively). Leukocytosis induced by H37Rv was similar to that induced by W4. The predominant leukocytes in the CSF were monocytes and lymphocytes (>90%).

Elevated numbers of colony-forming units and CSF leukocytosis were associated with local TNF production. CDC1551 initially induced higher mean levels of TNF; however, this response waned by 4 weeks after infection and then disappeared (figure 2B). In contrast, the TNF levels induced by HN878 and W4 were lower at the start of infection and then persisted in the CSF for 8 weeks. H37Rv induced very low to undetectable levels of TNF in the CSF; plasma TNF showed similar patterns (data not shown).

The inflammatory response was correlated with the clinical manifestations of TBM. No neurologic signs were seen in rabbits infected with CDC1551 or H37Rv (figure 2C), whereas rabbits infected with HN878 or W4 demonstrated significantly worse signs of disease beginning 3 weeks after infection, including loss of coordination, pareses, and paralysis of the limbs (P<.0001).

CNS pathologic changes in infected rabbits. Very mild focal inflammation of the meninges, with no vasculitis or necrosis and minimal mononuclear leukocytic infiltration, was induced by H37Rv (figure 3A and 3B). CDC1551 induced moderate focal inflammation with no vasculitis or necrosis, some distension of the subarachnoid space, and increased mononuclear leukocytic infiltration (figure 3C and 3D). In contrast, diffuse severe meningitis, with thickening of the leptomeninges and prominent necrotizing vasculitis, was observed after infection with W4 (figure 3E and 3F). In rabbits infected with HN878, the inflammatory infiltrate was most severe, with necrotizing granulomatous meningitis, encephalitis, and vasculitis within the cortex of the brain (figure 3G and 3H). Large granulomas with central necrosis, surrounded by macrophages, lymphocytes, and scattered polymorphonuclear cells, were noted. Nu-
Inflammatory response and clinical course of disease in rabbits infected intrathecally with HN878 or HN878\(\text{pks1-15::hyg}\). Next, rabbits were infected with HN878 or the PGL-deficient mutant, HN878\(\text{pks1-15::hyg}\), and were monitored for 8 weeks. By 1 week after infection, we observed a significant difference between the numbers of colony-forming units of HN878 and HN878\(\text{pks1-15::hyg}\) in CSF (\(P = .001\)) (figure 4A). Clearance of HN878\(\text{pks1-15::hyg}\) progressed faster, and no bacilli were detected from 5 to 8 weeks after infection. CSF leukocytosis induced by HN878\(\text{pks1-15::hyg}\) was significantly lower throughout the experiment (\(P = .01\)) (figure 4B). No TNF was detected in the CSF or plasma of rabbits infected with HN878\(\text{pks1-15::hyg}\); fewer neurologic signs and a significantly milder course of disease were noted (figure 4C). Only 1 rabbit infected with HN878\(\text{pks1-15::hyg}\) developed paresis of the hind limbs during week 8. Histopathologic examination of brain and meninges of rabbits infected with HN878\(\text{pks1-15::hyg}\) revealed mild focal meningeal inflammation with no vasculitis (data not shown). Both the inflammation and pathologic changes induced by HN878\(\text{pks1-15::hyg}\) were less severe, compared with that induced by HN878, even though \(>4\ \log_{10}\ \text{cfu}\) were observed in the brain (figure 4D).

When rabbits were infected with a mixture (1:1) of HN878 and HN878\(\text{pks1-15::hyg}\), viable bacilli persisted in the CSF throughout the experiment (figure 5A). PCR analyses of individual colonies showed that, beginning 2–4 weeks after infection, the predominant strain was HN878, representing \(>73\%\) of the total colony-forming units in the CSF. In the brain and lungs, HN878 represented 75\% and 89\% of the total colony-forming units, respectively. Similarly, infection of rabbits with a 1:1 mixture of HN878 and CDC1551 resulted in better control of the CDC1551 strain (figure 5B). In the CSF, brain, and lungs, the predominant strain by 4 weeks after infection was HN878 (100\% of colony-forming units in the lung).

DISCUSSION

We have demonstrated that \(M.\ \text{tuberculosis}\) clinical isolates are differentially virulent (as defined by severity of disease and/or bacillary load in infected tissues) in the rabbit. We have shown that W4 and HN878, both of which are members of the W/Beijing family of strains, are more virulent than CDC1551 and the control laboratory strain H37Rv. Infection with HN878 or W4 resulted in a persistent bacillary load in the CSF and higher bacillary loads in the brain; a propensity to disseminate to other tissues, such as lung and liver; and prolonged inflammation. Rabbits infected with HN878 or W4 developed severe progressive meningitis with loss of coordination and limb paralyses by 3 weeks after infection. The hypervirulence of HN878 numerous acid-fast bacilli were found within the macrophages of the necrotizing granulomas (figure 3G, insert).
Figure 5. Bacillary load in the central nervous system and lungs of rabbits infected intrathecally for 4 weeks with a mixture of Mycobacterium tuberculosis strains HN878 \( (n = 4) \) and HN878\( pks1-15::hyg \) \( (n = 4) \) \( (A) \) or strains HN878 \( (n = 8) \) and CDC1551 \( (n = 8) \) \( (B) \). Upper panels. No. of colony-forming units in the cerebrospinal fluid (CSF), based on polymerase chain reaction analyses of isolates. Middle panels. Ratio \( (\%) \) of HN878 (dark gray bars) versus HN878\( pks1-15::hyg \) or CDC1551 (checkered bars) in the CSF at each time point. Lower panels. Ratio \( (\%) \) of HN878 (dark gray bars) vs. HN878\( pks1-15::hyg \) or CDC1551 (checkered bars) in the brains and lungs of rabbits at 4 weeks after infection. No CDC1551 was detected in the lungs. Values are means \( \pm \) SEs.

appeared to be related to PGL, which is produced by this strain and W4 but not by CDC1551 or H37Rv [16]. Rabbits infected with HN878\( pks1-15::hyg \), which does not produce PGL, showed reduced pathologic changes and attenuated clinical manifestations that were more similar to those induced by CDC1551. Interestingly, rabbits infected with CDC1551, H37Rv, or HN878\( pks1-15::hyg \) did not show any (or showed only very limited) neurologic signs, in spite of the presence of a significant bacillary load in the brain.

Differences between the virulence of HN878 and that of the mutant HN878\( pks1-15::hyg \) strain have recently been reported in the mouse infection model [16]. Infection with HN878\( pks1-15::hyg \) was found to be associated with increased survival of the mice and improved host Th1 immunity, compared with that in mice infected with HN878 [16]. However, there was no reduction in the bacillary load in the lungs of mice infected with HN878\( pks1-15::hyg \). Differences between the various animal models often make it difficult to directly compare results from one model to another. However, the different models do provide alternative and complementary approaches to the study of TB. Our model of TBM does not fully reflect the natural history of the infection in humans. However, the model does resemble human TBM clinically and histopathologically and facilitates the study of bacterial factors that promote inflammation, which is especially prominent in the CNS. Mice are more resistant to M. tuberculosis infection than are humans and show no typical lung pathologic changes [19]. However, mice are ideal for the study of the host immune response to the bacilli. Guinea pigs are much more susceptible to M. tuberculosis infection than are humans, and even a single organism kills every animal [20]. Lung granulomas in these animals develop necrosis, as do some granulomas in human lungs. Nonhuman primates mimic human disease very well, probably better than any other model [21], but they are very expensive, and biocontainment facilities for the study of monkeys are few and limited in their capacity. In vitro human monocyte infection, although clearly very reductionist, provides the opportunity to perform selected functional assays and molecular analyses of signaling and gene expression after infection with different strains. To better understand TB, we must select the most relevant model for each experimental question pursued. Taken together, the results we have obtained using different infection models are consistent with the idea that HN878 is more virulent.
than CDC1551 and that this property is, at least in part, dependent on the production of PGL, a lipid that appears to subvert the activation of the host protective immune response [11, 13, 16, 22].

The results of our mixed-infection experiments suggest that, during the early response to low-dose CSF *M. tuberculosis* infection, the host cellular response to each strain develops and progresses independently (figure 5)—that is, macrophages infected with HN878 were not activated by their intracellular bacilli, and these macrophages remained permissive to the infection. In contrast, macrophages infected with CDC1551 or HN878*pksl-15::hgy* were activated by their intracellular bacilli, and these macrophages controlled the growth of the organisms. In addition, macrophages infected with HN878 that left the subarachnoid space and migrated into the brain supported the growth of the bacilli. Macrophages infected with CDC1551 or HN878*pksl-15::hgy* controlled the growth of the bacilli in the brain. Similarly, macrophages infected with HN878 breached the BBB and seeded the lung more efficiently than did macrophages infected with CDC1551 or the mutant strain. Thus, the innate immune response to infection with one strain did not appear to affect the innate response to the other strain during coinfection. Since we examined only the early events during infection in these studies, we cannot predict the nature of the acquired immune response to mixed infection.

Clearly, TNF-α is required for protection against *M. tuberculosis* [23, 24]. The most clinically relevant example of this requirement is the observation that neutralization of TNF-α by anti-TNF monoclonal antibodies in patients with rheumatoid arthritis can induce reactivation of latent TB [25–27]. Our recent studies in mice and in human monocytes in vitro have characterized the immunologic determinants associated with an efficient Th1 protective response against *M. tuberculosis* infection. In addition to TNF-α, other cytokines and chemokines, including IL-12, macrophage inflammatory protein-3α, chemokine receptor CCR-7, IL-6, and Fas-ligand, appear to be required for a protective response. These mediators are induced suboptimally during the early interaction of HN878 with human or mouse macrophages, compared with the interaction of CDC1551 or HN878*pksl-15::hgy* with the cells [16, 22]. In the present study, too, rabbits infected with CDC1551 demonstrated an early peak in TNF levels in the CSF and plasma, followed by accelerated clearance of the bacilli from the CSF. However, the presence of TNF was not sufficient to provide protection, as was demonstrated by the fact that both HN878 and W4 induced TNF production in the CSF but were not cleared. Since reagents are not yet available for the measurement of other immune-regulatory mediators in the rabbit, we have not determined their levels. However, we predict that the other cytokines and chemokines associated with optimal Th1 protective responses are not efficiently induced in the rabbit in response to HN878 and W4 infection.

Even in the absence of immunologic reagents, the rabbit CNS infection model is useful for the study of pathogenesis and disease, because of the excessive sensitivity of the brain to inflammatory and/or immune perturbations. Although TNF-α is protective against *M. tuberculosis* infection, it is known to be detrimental to the host when present in excess and for a long time, especially in the brain [8]. The clinical signs observed after intrathecal infection with W4 or HN878 were most likely a result of tissue damage in the brain, associated with prolonged bacillary load, leukocytosis, and persistent TNF production. One of the activities of TNF is to induce coagulation [28] and formation of thrombi, leading to vascular occlusions and necrosis. In the present study, histopathologic examination of the brains of rabbits infected with HN878 or W4 demonstrated directly the impairment of the vasculature. A possible mechanism leading to endarteritis and tissue damage is TNF-induced production of nitric oxide synthase, nitric oxide, and free radicals [29–32]. It is also known that proinflammatory cytokines in the CNS alter the BBB and cause enhanced adhesion-molecule expression on the microvasculature [33]. This activation of endothelial cells can lead to production of proinflammatory cytokines, with an influx of monocytes and T cells into the tissues and intensification of the endothelial damage [34, 35]. Injection of TNF-α directly into the neural parenchyma in rats induces formation of inflammatory infiltrates around the injection site [36, 37]. In addition, recombinant human TNF-α injected into the cisterna magna of rabbits causes a reduction in cerebral oxygen uptake and lower cerebral blood flow [29]. When capillaries are presensitized by mycobacterial products, even very small amounts of TNF-α can produce deleterious effects [38]. These activities of TNF may, in part, drive the pathogenesis of TBM, since the most common reason for the cranial nerve palsy, pareses, and paralyses is occlusions of large and small vessels. Since the major sources of TNF in the CNS are newly recruited blood monocytes and resident microglial cells, it is likely that these cells are more sensitive and/or responsive to certain mycobacterial components, especially in the rabbit.

In the present study, the PGL-deficient strain HN878*pksl-15::hgy* did not induce any TNF production in the CSF and caused very limited signs of disease. Thus, this mutant was more like CDC1551 than the parental HN878 strain. Our results demonstrate clearly that *M. tuberculosis* PGL is involved in the induction of CNS inflammation and tissue damage in infected rabbits and contributes significantly to *M. tuberculosis* virulence. However, the contribution of PGL notwithstanding, our results and those of others suggest that the virulence of *M. tuberculosis* is not determined by a single microbial gene but is the outcome of a complex, dynamic interaction between host and microbial properties [39]. For example, a number of recent studies have
suggested that other surface lipids, including the sulfolipid SL1 [40, 41], lipoarabinomannan [42], the 19-kDa lipoprotein [43], and others [44], contribute to virulence of mycobacteria. Polymorphisms among \textit{M. tuberculosis} strains are more extensive than initially anticipated. Several differences between the genomes of the laboratory strain \textit{M. tuberculosis} H37Rv, which has a long history of passage, and the clinical isolate CDC1551 were recently reported [45]. The genome of another \textit{M. tuberculosis} clinical isolate (strain 210), which has an IS6110-based restriction fragment length polymorphism pattern identical to that of HN878 [22], is in the process of being sequenced at The Institute of Genome Research, and early results suggest that it differs significantly from the genome of CDC1551 [46].

Many of these genomic differences might contribute to differences in immunogenicity and pathogenicity among \textit{M. tuberculosis} clinical isolates. In the future, the ability to combine genomic information with pathogenesis studies employing diverse clinical strains, such as those described in the present article, will enable investigators to continue to unravel the molecular basis of \textit{M. tuberculosis} virulence.

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**References**

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