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ORIGINAL RESEARCH

# Diversity of *glpK* Gene and Its Effect on Drug Sensitivity in *Mycobacterium bovis*

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**Background:** Glycerol kinase (*glpK*) is essential for the first step of glycerol catabolism in *Mycobacterium tuberculosis*. However, *Mycobacterium bovis* has been known to grow poorly in glycerol media because of a base insertion in the *glpK* gene.

**Methods:** We analyzed the *glpK* gene sequences of 60 clinical *M. bovis* isolates, and determined the minimum inhibitory concentration of 14 drugs by microdilution method to evaluate the effect of frameshift mutations on drug sensitivity. The effect of *M. bovis* growth rate on its drug sensitivity was investigated using bacteria grown on glycerol or pyruvate.

**Results:** A total of 44 (73.33%) clinical *M. bovis* isolates have frameshift mutations in a homopolymeric tract of 7 cytosines in the *glpK* gene. 15.00% *M. bovis* isolates showed phenotypic drug resistance. Glycerol metabolism-deficient *M. bovis* showed reduced susceptibility to 9 out of 14 tested drugs. Mutations in the *glpK* gene can lead to impaired growth in glycerol-based media, while the minimal inhibitory concentration values of slow-growing *M. bovis* were higher.

**Conclusion:** Mutations in the *glpK* gene can lead to slowed growth and reduced susceptibility to drugs in *M. bovis*, which may contribute to the emergence of drug-resistant *M. bovis* and pose a threat to human health owing to the zoonotic capacity of *M. bovis*.

**Keywords:** *Mycobacterium bovis*, tuberculosis, *glpK*, drug resistance

## Introduction

Tuberculosis (TB), a communicable disease, ranks as the second leading cause of death from a single infectious agent, after COVID-19 in 2020, with roughly a quarter of the world's population infected with *Mycobacterium tuberculosis*.[1] Since 1990, TB mortality has decreased; nevertheless, the rise of multidrug-resistant (MDR) and extremely drug-resistant (XDR) strains of *M. tuberculosis* represents a serious public health threat.[1–3] Unlike drug-sensitive TB, which can be treated by 6 months of chemotherapy with the current four-drug frontline regimen, MDR-TB requires at least 18 to 24 months of therapy with four to six drugs, including fluoroquinolone and one injectable agent.[2,4,5]

The *M. tuberculosis* complex is a group of closely genetically related yet phenotypically diverse organisms.[6] A range of in vitro characteristics can be used to distinguish the members of the complex; for example, unlike *M. tuberculosis, M. bovis* is unable to utilise glycerol as its sole carbon source.[7] *M. bovis* primarily infects cattle, but can also infect a wide range of species, including humans.[8] The genomes of *M. bovis* and *M. tuberculosis* are more than 99.95% identical, and the symptoms of human disease caused by *M. bovis* are similar to those of disease caused by *M. tuberculosis*.[9,10] Treatment of disease caused by *M. bovis* usually includes rifampicin, isoniazid, and ethambutol.[11] Due to the exclusion of pyrazinamide, since all strains of *M. bovis* are resistant to it, treatment duration is generally extended to 9 months.[12] There have been some reports of infections caused by MDR *M. bovis*, including both sporadic cases and transcontinental outbreaks.[13–17]

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The 2020 WHO global tuberculosis report estimated that there were 140,000 new human cases of zoonotic tuberculosis caused by *M. bovis* globally in 2019.[3] The use of culture media such as glycerol, that inhibit *M. bovis* growth, has led to the number of human cases of *M. bovis* infection being underestimated and unreported.[18–20] A frameshift mutant in the *glpK* gene that encodes the glycerol kinase enzyme of *M. bovis* causes the glycerol catabolic defect.[7] Growing evidence suggests that frameshift mutations in a homopolymeric tract (HT) of 7 cytosines (7C) in the *glpK* gene lead to drug tolerance in *M. tuberculosis*.[21,22]

Here, we focus on the *glpK* frameshift mutations in the clinical isolates of *M. bovis*. We found that multigene mutation patterns in the 7C HT of the *glpK* gene in *M. bovis* and not all frameshift mutations caused glycerol kinase inactivation. Glycerol has a promotive effect on the growth of a part of *M. bovis*. Frameshift mutations that disrupt glycerol kinase activity contribute to an extensive reduction in antibiotic sensitivity in bovine tuberculosis.

# Materials and Methods

## Bacterial Strains and Culture Conditions

Clinical *M. bovis* strains are stored at the Chinese Center for Disease Control and Prevention. The clinical *M. bovis* isolates used in this study were isolated from a large cattle slaughterhouse in Xinjiang, China. Suspected *M. bovis* infected tissue samples were collected, and strain isolation and identification were all performed in the biosafety level 3 (BSL-3) laboratory. Farm animal welfare standards were met during transport and slaughter of the cattle. Transport drivers and escorts were trained in basic veterinary as well as animal welfare related knowledge. The slaughterhouse used humane slaughter, and the slaughterers were trained in animal welfare-related knowledge.

The clinical strains used in this study were isolated from slaughtered cattle. This study does not involve research studies on humans or animal experiments, so ethical approval for this study was not needed.

Unless otherwise stated, the *M. bovis* strains were cultivated at 37°C either in Middlebrook 7H9 broth (Difco) containing 0.05% Tween 80 or on Middlebrook 7H10 agar supplemented with 0.2% pyruvate, both enriched with 10% oleic acid-albumin-dextrose catalase (Difco). To test the growth of *M. bovis* in glycerol-based media, 0.2% glycerol was used instead of pyruvate.

## The Minimal Inhibitory Concentration (MIC) Determination

MICs for clinical *M. bovis* strains were determined by the microdilution method, as described previously.[23] Briefly, the first column of wells of a 96-well plate (Costar) was filled with 200 µL of 7H9 containing the drug at its maximum concentration to be tested. The remaining wells were filled with 100 µL 7H9 medium. This was used to perform 2-fold serial dilution of the drugs, Bedaquiline (BDQ; 0.0156 to 2 µg/mL), Amikacin (AMK; 0.25 to 32 µg/mL), Ethambutol (EMB; 0.125 to 16 µg/mL), Isoniazid (INH; 0.025 to 3.2 µg/mL), Levofloxacin (LEV; 0.0125 to 1.6 µg/mL), Moxifloxacin (MXF; 0.0625 to 8 µg/mL), Delamanid (DLM; 0.0156 to 2 µg/mL), Linezolid (LZD; 0.03125 to 4 µg/mL), Clofazimine (CFZ; 0.0625 to 8 µg/mL), Rifampin (RIF; 0.0625 to 8 µg/mL), Rifabutin (RFB; 0.0625 to 8 µg/mL), Para-aminosalicylic acid (PAS; 0.125 to 16 µg/mL), Ethionamide (ETH; 0.25 to 32 µg/mL), Kanamycin (KAN; 1 to 128 µg/mL). The plates were incubated at 37°C for 14 days and scored as either growth or no growth. The MIC was defined as the concentration at which no microbial growth was observed visually.

MIC for *M. bovis* C68004 was performed similarly as above, using 7H9 medium supplemented with 0.2% glycerol or pyruvate.

## RNA Isolation and Real-Time Quantitative PCR

RNA was extracted as described previously.[24] First-strand cDNA synthesis was performed by the HiScript III 1st Strand cDNA Synthesis Kit (Vazyme). Real-time quantitative PCR was performed on the CFX96 RealTime Thermal Cycler (Bio-Rad) with AceQ qPCR SYBR Green Master Mix (Vazyme). The thermal cycling conditions were initial denaturation for 5 min at 95°C, followed by 40 cycles of 95°C for 10s, 56°C for 30s and 72°C for 30s. Fluorescence measurements were recorded at each annealing step. A melting curve analysis was performed to ensure the specificity of the products.

## Statistical Analysis

All assays were performed on 3 separate occasions. The results were expressed as means with standard errors of the mean. Statistical significance was determined by using Unpaired *t*-test (two-tailed).

# Results

## Multiple Mutation Patterns Exist in the *glpK* Gene of Clinical *M. bovis*

When glycerol is the sole carbon source, one of the key in vitro distinctions between *M. bovis* and *M. tuberculosis* is the requirement for pyruvate.[25] *M. bovis* AF2122/97 has a single C insertion in the *glpK* 7C HT, causing a frameshift and leading to a truncated coding sequence.[7] Noteworthy, in BCG, a 2 bp insertion in the *glpK* 8C HT of the *M. bovis* AF2122/97 *glpK* corrects the frameshift and results in an extra codon with respect to the *M. tuberculosis glpK* (Table 1). This extra codon was discovered in all *M. bovis* BCG strains tested (Pasteur, Tokyo, Danish, Russia, Tice, Frappier, Sweden), which can thrive solely on glycerol.[6] Further analysis revealed that *M. microti* OV254, had the same frameshift mutation in *glpK* as *M. bovis* AF2122/97 (Table 1). *M. microti*, like *M. bovis*, requires the addition of pyruvate to glycerinated medium for growth.[6]

To explore the diversity of *glpK*, we sequenced 60 clinical isolates of *M. bovis* and grouped them according to *glpK* 7C HT (Table 2). *GlpK* frameshift mutations are common in *M. bovis* isolates. 40.00% of clinical *M. bovis*, like *M. bovis* AF2122/97, had glycerol kinase inactivation due to a single C insertion in the *glpK* 7C HT. Moreover, we detected 26.67% of clinical isolates with *glpK* 7C HT, the same as *M. tuberculosis* H37Rv. In addition, we detected 26.67% of *glpK* 9C HT isolates and 3.33% of *glpK* 10C HT and *glpK* 11C HT isolates (Table 2).

Next, we evaluated the frequency of frameshift mutations in the *glpK* 7C HT in *M. tuberculosis*. We counted the genomic information on 2819 *Mycobacterium tuberculosis* strains in GMTV database[26] and found that frameshift mutations in the *glpK* 7C HT are also present in *M. tuberculosis*, but at a lower frequency than in *M. bovis* (Table 3).

## Glycerol Catabolic Mutations are Associated with the Growth of *M. bovis*

Since the *glpK* gene encodes an enzyme involved in glycerol metabolism (Supplementary Figure 1), and early studies concluded that the growth of *M. tuberculosis* was strongly promoted by the addition of glycerol to the medium during in vitro culture,[27,28] we monitored the growth curves of different *M. bovis glpK* HT strains. Similar to *M. tuberculosis* H37Rv, the growth of *M. bovis glpK* 7C HT strain was promoted in the glycerol-based medium compared to pyruvate (Figure 1A). In contrast, the *M. bovis glpK* 8C HT strain, representing the majority of *M. bovis*, grew slowly in the

**Table 1** *GlpK* Slippage Site Sequences of Several Mycobacterial Species

| Mycobacterial Strains | *GlpK* Slippage Site Sequences |
|---|---|
| *M. tuberculosis* H37Rv | 565 GCCCCCCCA 573 |
| *M. bovis* AF2122/97 | 565 GCCCCCCCCA |
| *M. bovis* BCG Pasteur 1173P2 | 565 GCCCCCCCCCCA |
| *M. microti OV254* | 565 GCCCCCCCCA |
| *M. orygis* 51145 | 565 GCCCCCCCA |
| *M. canettii CIPT* 140010059 | 565 GCCCCCCCA |

**Table 2** 60 Clinical *M. bovis* Isolates Were Grouped According to the *glpK* Slippage Site Sequences

| *GlpK* HT | Sequences | Number of Isolates | Proportion of Isolates |
|---|---|---|---|
| 7C | 565 GCCCCCCCA 573 | 16 | 26.67% |
| 8C | 565 GCCCCCCCCA | 24 | 40.00% |
| 9C | 565 GCCCCCCCCCA | 16 | 26.67% |
| 10C | 565 GCCCCCCCCCCA | 2 | 3.33% |
| 11C | 565 GCCCCCCCCCCCA | 2 | 3.33% |

**Table 3** 2816 Clinical *M. tuberculosis* Isolates Were Grouped According to the *glpK* Slippage Site Sequences

| *GlpK* HT | Sequences | Number of Isolates | Proportion of Isolates |
|---|---|---|---|
| 7C | 565 GCCCCCCCA 573 | 2791 | 99.11% |
| 8C | 565 GCCCCCCCCA | 20 | 0.71% |
| 10C | 565 GCCCCCCCCCCA | 4 | 0.14% |
| 11C | 565 GCCCCCCCCCCCA | 1 | 0.04% |

glycerol-based medium (Figure 1B). Furthermore, the *M. bovis glpK* 10C HT strain makes good use of glycerol, probably because this mutation only inserts an extra codon and does not cause extensive codon mismatches (Figure 1C).

To further verify the glycerol utilization ability of *M. bovis glpK* 10C HT strain, we next observed its growth in solid medium with glycerol or pyruvate as carbon sources. On the 12th day of incubation, lots of colonies were visible on 7H10 agar plates supplemented with 0.2% glycerol (Figure 1D), while a few colonies were visible on 7H10 agar plates supplemented with 0.2% pyruvate on the 14th day (Figure 1E).

*M. bovis*, an obligate aerobe, induces the expression of *narX*,[29] *narK2*,[30] *dosR*,[31] *hspX*,[32] and *frdA*[33] in response to hypoxia. To measure the growth of the *glpK* 10C HT strain in both media, we monitored the transcript levels of these genes. Due to the fast growth rate in glycerol medium, the *glpK* 10C HT strain consumed oxygen more rapidly and the hypoxia-induced *narX, narK2, dosR, hspX*, and *frdA* expressions were elevated (Figure 1F).

## *glpK* Frameshift Mutations Can Affect Anti-Tuberculosis Drug Sensitivity in *M. bovis*

To investigate the drug resistance of clinically popular *M. bovis*, we conducted drug susceptibility testing (DST) for 60 isolates from China. We detected 9 isolates (15% of total) showed phenotypic resistance to the tested drugs. Among these, 4 (6.67%) were resistant to delamanid, 4 (6.67%) were resistant to kanamycin, 2 (3.33%) were resistant to ethionamide, and 1 (1.67%) was resistant to isoniazid. Moreover, 2 (3.33%) isolates were resistant to two drugs at the

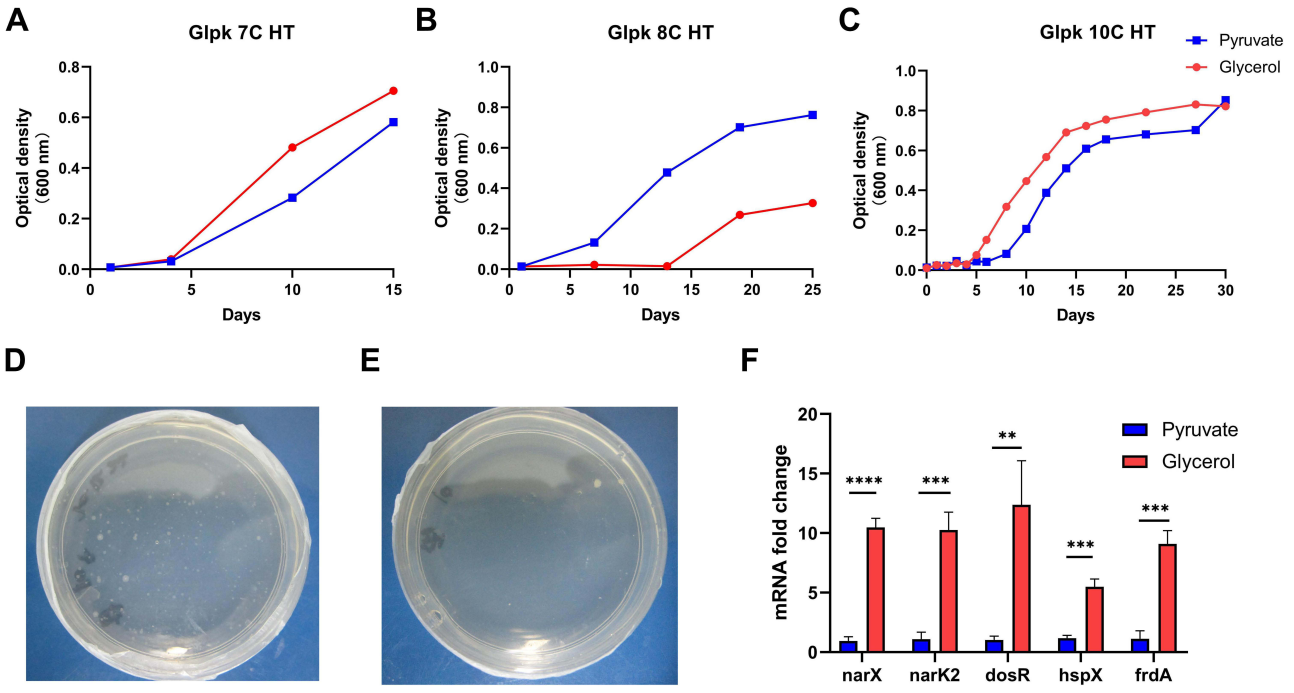


**Figure 1** Glycerol promotes the growth of *M. bovis glpK* 7C HT strain and *M. bovis glpK* 10C HT strain in vitro. (**A**–**C**) Growth curves of *M. bovis glpK* 7C HT strain, *glpK* 8C HT strain and *glpK* 10C HT strain in glycerol or pyruvate medium. (**D**) Colony formation of the *M. bovis glpK* 10C HT strain on day 12 of growth in glycerol solid medium. (**E**) Colony formation of the *M. bovis glpK* 10C HT strain on day 14 of growth in pyruvate solid medium. (**F**) The relative expression of mRNA in glycerol and pyruvate medium for the *M. bovis glpK* 10C HT strain was compared. **Significant at $\rho < 0.01$; ***Significant at $\rho < 0.001$; ****Significant at $\rho < 0.0001$ were determined by unpaired *t*-test (2 tailed).

**Table 4** Qualitative Classification and MIC (Expressed in μg/mL, with Respective Cut-off Points) for DST in *M. bovis* Isolated from China

| Isolate | *GlpK* HT | Classification | | | | MIC (μg/mL) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | DLM | KAN | ETH | INH | DLM (0.2) | KAN (2.5) | ETH (5.0) | INH (0.2) |
| B11 | 7C | S | S | **R** | S | ≤0.015 | ≤1 | **8** | 0.1 |
| B13 | 7C | **R** | S | S | S | **0.25** | 2 | 2 | 0.2 |
| B17 | 7C | **R** | S | S | S | **0.25** | 2 | 4 | 0.1 |
| B23 | 8C | S | **R** | S | S | ≤0.015 | **4** | 2 | 0.1 |
| B29 | 8C | S | S | **R** | S | ≤0.015 | ≤1 | **8** | 0.1 |
| B38 | 8C | **R** | **R** | S | S | **>1** | **8** | 2 | 0.1 |
| B42 | 8C | **R** | S | S | **R** | **>1** | 2 | 4 | **0.4** |
| B48 | 9C | S | **R** | S | S | 0.03 | **4** | 4 | 0.1 |
| B55 | 9C | S | **R** | S | S | ≤0.015 | **4** | 2 | 0.1 |

**Note**: Qualitative classification and MIC of resistant strains are shown in bold.
**Abbreviations**: S, sensible; R, resistant; DLM, delamanid; KAN, kanamycin; ETH, ethionamide; INH, isoniazid.

same time (Table 4 and Supplementary Table 1). However, we did not find any known drug-resistance gene mutations in these phenotypic resistant isolates (Supplementary Table 2).

Frameshift mutations in *glpK* 7C HT leading to drug tolerance in *Mycobacterium tuberculosis* have been reported.[21,22] We analyzed the MICs of different *glpK* HT *M. bovis* strains and found that *glpK* 9C HT strains showed lower drug sensitivity to 9/14 of the assessed drugs, including amikacin, moxifloxacin, para-aminosalicylic acid, rifampin, ethambutol, rifabutin, levofloxacin, clofazimine, and linezolid (Figure 2A–I). In addition, the levofloxacin sensitivity test showed a reduction in the sensitivity of *glpK* 8C HT strains (Figure 2G). However, we found no significant difference in the MIC level of five drugs, including isoniazid and bedaquiline (Supplementary Figure 2).

Since the MIC was measured in glycerol liquid medium, in which *M. bovis glpK* 8C HT strains and *M. bovis glpK* 9C HT strains grew slowly compared to *M. bovis glpK* 7C HT strains, next, we evaluated the effect of growth rate on drug susceptibility.

## The Growth Rate of *M. bovis* Has an Impact on Drug Susceptibility

We used *M. bovis* C68004, a *glpK* 10C HT strain, and tested its drug MIC using glycerol or pyruvate-based medium. Among the 12 drugs tested, the MIC of 7 drugs in glycerol-based medium was lower than that in pyruvate-based medium (Table 5). This indicates that *M. bovis* C68004 is more sensitive to drugs due to its fast growth rate in glycerol-based medium.

# Discussion

Unlike most bacterial pathogens, mycobacteria are capable of utilizing multiple carbon substrates.[34,35] Early studies indicated that glycerol-fed *M. tuberculosis* grows faster and the bacilli reach a higher density than when metabolizing glucose or fatty acids, leading to the use of glycerol in virtually all standard mycobacterial growth media.[27,36] However, due to frameshift mutation in a homopolymeric tract of 7 cytosines in the *glpK* gene, glycerol metabolism in *M. bovis* is defective and substituted it with pyruvate.[7,25]

Our sequence analysis of the *glpK* gene of clinical *M. bovis* revealed that 30% of *M. bovis* (*glpK* 7C HT strains and *glpK* 10C HT strains) can be subjected to the growth-promoting effect of glycerol. Therefore, we recommend testing for *glpK* gene status when performing *M. bovis* culture and selecting the appropriate medium.

*M. bovis*, the main cause of bovine tuberculosis (bTB), can cause major economic problems worldwide and can infect humans, posing a threat to public health.[8] While the DSTs of *M. tuberculosis* isolated from human cases are generally

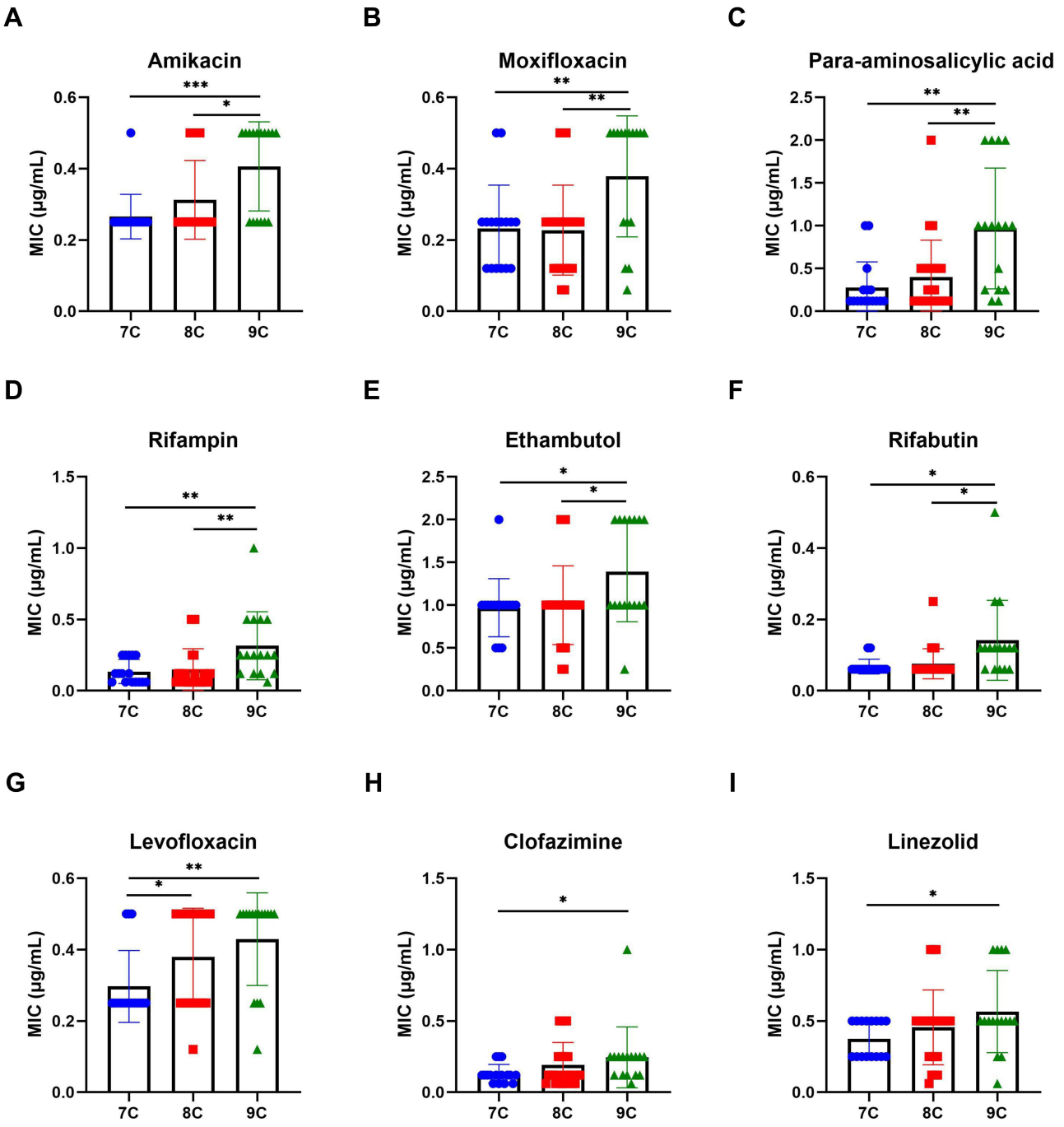


**Figure 2** The *glpK* mutation contributes to the anti-tuberculosis drug tolerance capability of *M. bovis* isolates. (**A**–**F**) MIC assay was conducted to investigate the drug sensitivity of different mutants of *glpK* of *M. bovis*. The sensitivity of (**A**) Amikacin, (**B**) Moxifloxacin, (**C**) Para-aminosalicylic acid, (**D**) Rifampin, (**E**) Ethambutol, (**F**) Rifabutin, (**G**) Levofloxacin, (**H**) Clofazimine, and (**I**) Linezolid was determined by MIC assay. *Significant at $\rho < 0.05$; **Significant at $\rho < 0.01$; ***Significant at $\rho < 0.001$ were determined by unpaired *t*-test (2 tailed). Due to the low number of *glpK* 10C HT and *glpK* 11C HT strains, they were not analyzed.

assessed, research on antitubercular DSTs of *M. bovis* isolated from animals is limited.[12] Our data showed that 15% of the *M. bovis* isolates are resistant to at least one drug. We detected phenotypic resistance to delamanid, kanamycin, ethionamide, and isoniazid in *M. bovis*, yet no drug-resistance gene mutations were found. In another study of drug resistance in *M. bovis*, a similar phenomenon was found. In Brazil, despite the government's prohibition on the treatment of infected cattle, 31.3% of *M. bovis* showed resistance to the tested drugs, and 16% were classified as MDR *M. bovis*.[18] Although it may be influenced by multiple factors, Mycobacteria sp. resistance to antimicrobials is often associated with

**Table 5** MIC of *M. bovis* C68004 in Glycerol or Pyruvate-Based Medium MIC (μg/mL)

| | OFL | RIF | AMK | MXF | KAN | SM | PAS | ETH | CS | INH | ETH | RFB |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Glycerol | 0.5 | 0.25 | 0.25 | 0.5 | 1.25 | 0.5 | 2 | 1.25 | 16 | 0.063 | 4 | 0.25 |
| Pyruvate | 1 | 0.5 | 0.5 | 0.5 | 1.25 | 0.5 | 4 | 2.5 | 32 | 0.125 | 4 | 0.125 |

**Abbreviations**: OFL, ofloxacin; RIF, rifampin; AMK, amikacin; MXF, moxifloxacin; KAN, kanamycin; SM, streptomycin; PAS, para-aminosalicylic acid; ETH, ethionamide; CS, cycloserine; INH, isoniazid; EMB, ethambutol; RFB, rifabutin.

mutations in target-encoding or related genes.[35] However, the resistant strains were not subjected to sequencing of rpoB, katG, or the promoter region of inhA gene.[18]

The use of subtherapeutic doses of antibiotics as growth promoters, which is a commonly applied method to maximize yield in animal production, has been linked to the rise of antimicrobial resistance and cross-resistance.[34] However, in many countries, first and second line antibiotics against TB are not approved for animal consumption.[28] This further complicates the identification of the sources implicated in the acquisition of mutations conferring resistance to these drugs since multiple factors could participate in this process. Canonical mechanisms of resistance are generally grouped into three broad categories: target modification, drug inactivation, and drug transport.[37–39] There is growing evidence that metabolic mutations also contribute to the evolution of bacterial drug resistance.[39–41] Indeed, metabolic adaptation may represent a class of resistance mechanisms whereby, beyond conferring tolerance, cells alter their metabolic response to lessen antibiotic lethality's downstream toxic effects.[39]

Our finding showed that fast-growing *M. bovis* is more sensitive to antibiotics. *M. tuberculosis* greatly reduces growth and metabolic activity in chronically infected animals.[42–44] This means that mycobacteria that are sensitive to drugs in DSTs may not be effectively killed or limited by drugs in vivo, which undoubtedly promotes the emergence of drug-resistant strains. This is consistent with previous work in which virtually all antibiotics preferentially kill rapidly replicating bacteria.[42,45,46]

*GlpK* encodes glycerol kinase, which is involved in the first step of glycerol catabolism. However, most *M. bovis* is defective due to frameshift mutations in *glpK* 7C HT. The average sequence divergence between *M. tuberculosis* and *M. bovis* is less than 0.05%.[47] In *M. tuberculosis*, similar frameshift mutations are present. Both in *M. bovis* and *M. tuberculosis*,[21,22] *glpK*-deficient strains have lower drug sensitivity than *glpK* 7C HT strains. We hypothesize that the use of subtherapeutic doses of antibiotics as growth promoters in cattle production has allowed *glpK*-deficient strains to be screened, leading to the prevalence of clinical *M. bovis glpK* 8C HT strains and *M. bovis glpK* 9C HT strains. Glycerol assimilation can alter growth rate, metabolism, and cellular structure.[21] We hypothesize that frameshift mutations that alter glycerol kinase activity may lead to decreased tricarboxylic acid cycle activity in favor of lipid anabolism. Increased lipid anabolism contributes to cell wall thickening, which reduces sensitivity to most anti-tuberculosis drugs.[48] Furthermore, it has also been suggested that GlpK is a member of the ROK (repressor, open-reading frame, kinase) protein family; thus, GlpK (like other sugar kinases) may potentially act as a transcription regulator and is linked to the stress response.[22]

*M. bovis* TB is clinically, pathologically, and radiologically indistinguishable from *M. tuberculosis*.[49] Moreover, there is growing evidence of the epidemic of MDR *M. bovis*, resulting in significant economic losses and threats to human health.[13,14,28,50–52] Rifampicin, isoniazid, and ethambutol are used to treat disease due to *M. bovis* in humans. Treatment duration is generally extended to 9 months due to the exclusion of pyrazinamide, since all strains of *M. bovis* are resistant to it.[11,12] Clinically prevalent *M. bovis* are mainly *glpK* 8C HT strains and *glpK* 9C HT strains with much lower susceptibility to drugs, which undoubtedly makes treatment more difficult.

In summary, we found that a proportion of *M. bovis* is subjected to the growth-promoting effect of glycerol, and fast-growing *M. bovis* is more sensitive to the drug. Frameshift mutations in *M. bovis glpK* HT are associated with lower drug sensitivity. Therefore, we need to thoroughly understand the relationship between bacterial metabolism and antibiotic function. Because *M. bovis* has the ability to infect both cattle and humans, the overuse of antibiotics in cattle can promote the development of drug resistance. Therefore, the use of antibiotics should be reduced to prevent human health threats from drug-resistant *M. bovis*.

## Conclusion

In clinical *M. bovis* isolates, frameshift mutations in a homopolymeric tract of 7 cytosines in the *glpK* gene are prevalent, leading to slowed growth and reduced susceptibility to drugs in *M. bovis*, which may contribute to the emergence of drug-resistant *M. bovis* and pose a threat to human health owing to the zoonotic capacity of *M. bovis*.

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## Disclosure

The authors report no conflicts of interest in this work.

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