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Efficacy of Tellurite Towards the Development of *Mycobacterium Tuberculosis* Drug-Resistant and Impact on their Drug Efflux Pump

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ABSTRACT:

Genotypic resistance refers to the presence of specific genetic mutations or variations within an organism's genome that confer resistance to certain treatments or interventions. This study focuses on the genotypic resistance of *Mycobacterium tuberculosis* through line probe assay. The virulent impact of Tellurite against *Mycobacterium tuberculosis* (or MBT) was checked by the growth of the cells in various tellurite concentrations (1 to 5 mM). The morphological outcomes of tellurite and metal uptake within the microbial cells were confirmed through the usage of a scanning electron microscope (SEM) and energy-dispersive X-ray analysis (EDX). The variation in the fold expression of the efflux pump gene was determined through the usage of RT-PCR. The bacterial strain was identified to be XRD-TB on the ground of genotypic resistance of isoniazid and rifampicin along with the resistance of second-line injectable and fluoroquinolones. XRD-TB exhibited black colonies in the presence of tellurite and their proliferation was held for up to 3 weeks as compared to the control set of experiment. The diminished size, and accumulation of metal, along with the characteristic peak of tellurite peaks emerged in the cells which were treated with metal. The MIC value of the MBT was found to be around 1 mM and was also found to have high susceptibility towards the elevated concentrations within the range of 2-5 mM. On the contrary, no potential metal inhibitory impact on the mmpl7 efflux system was recorded. Tellurite indicated a notable reduction in growth against the XDR-TB strain. However, the actual mechanism of this action needs to be explicated through further exhaustive research which can be considered as the potential future scope of this study.

KEYWORDS: *Mycobacterium tuberculosis*; Drug efflux pump; Tellurite; genotype resistance; *mmpl7* gene.

INTRODUCTION:

Tellurium is an essential biological micronutrient that exists in multiple states like tellurate, telluride, as well as tellurite in the state TeO^{2-}. Although, the toxicity potential of the tellurium has not been defined properly, however, the tellurite (Te) oxyanions have been reported to be highly toxic at even very low concentrations of 1µg/ml for almost all the microorganisms. The literature indicated that Te has been frequently used as an antimicrobial agent in order to treat various microbial infections (Cunha et al. 2009). Such effects of Te may be corroborated by the oxidant nature of Te by generating reactive oxygen species (ROS) like hydrogen peroxide, superoxide anion, hydroxyl radical, etc. Previously reported studies have also indicated that the role of glutathione (GSH) is also linked to tellurite toxicity. The GSH pool is oxidized in the presence of the tellurite and this phenomenon has been reported to generate superoxide radicals (Kessi et al. 2022). The reactive oxygen species might have an impact on a spectrum of macromolecules as well as various metabolic pathways. Apart from that, various other mechanisms of tellurite toxicity have also been reported that encompass Te-induced DNA damage, and translational arrest through cellular ATP depletion as histone phosphorylation of initiation factors as well as histones (Juan et al. 2021).

In spite of the relatively lower abundance of Te in nature along with higher toxicity, the microorganisms have been reported to have developed resistance towards tellurite (Te^R). The elevated and injudicious use of tellurite in various industries like rubber, electronics, metallurgy, and optics have in turn posed potential menaces of elevated environmental contaminations (Coral et al. 2006). This has in turn resulted in the isolation of different tellurite-resistant microbial species from the environment and other clinical specimens. The microorganisms have been reported to exhibit black deposits within their cells when tellurite is present within them. The tellurite gets accumulated within its cytosolic component. Such reduction may happen due to the activity of various enzymes like oxidase, catalyse, nitrate reductase, etc. to name a few. The Te^R determinants are found within the plastids, and chromosomes of the microorganisms (Zannoni et al. 2007). A few of the genes which have been found to be associated with this activity of tellurite resistance are *kil*A, *teh*AB, *ter*C, etc. Moreover, the gene-encoding enzymes have been found to be associated with the oxidative stress response (like *kat*G, *ahp*CF), multidrug efflux pump (like *acr*AB), metalloid efflux (like *ars*ABC), etc are also involved in the tellurite resistance of the microorganisms (Liu 2000).

After an exhaustive literature search, the authors discovered that very limited research has been conducted in this particular field of tellurite drug resistance against the bacteria *Mycobacterium tuberculosis*. A spectrum of mechanisms contributes to the appearance of drug resistance in tuberculosis (Azadi et al. 2018). On a broader scale, the intrinsic and extrinsic factors are linked to the promotion of resistant strains of *Mycobacterium tuberculosis.* The intrinsic factors have been found to be linked with the resistance mechanisms of the microbes as the result of genetic mutation, lower drug permeability through the cell membrane, drug target enzyme modification, presence of efflux pumps, and drug inactivation. The efflux pumps are also reported to be known as transporters (Behar et al. 2014). The microbial efflux pumps can be classified under five different heads which are as follows; i. small multidrug resistance (SMR), ii. ATP binding cassette (ABC), iii. Multidrug and toxic compound extrusion (MATE), iv. Major facilitator superfamily (MFS), and v. resistance nodulation division (RND). Combinedly all these efflux pumps contribute around 20% of all the efflux pumps found within the bacterial systems (Lamut et al. 2019).

Accordingly, the aim of this present study was focussed to investigate the tellurite toxicity against extensively drug-resistant (XDR) *Mycobacterium tuberculosis* strain. Furthermore, the inhibitory impact of tellurite on drug efflux pumps was also investigated as the extended aim of this study which in turn indicates the novelty of the present study.

MATERIALS AND METHODS

Strain Isolation and Characterization

The isolation of mycobacterial strain was isolated from the clinical sputum specimen. The specimen was characterized and processed for genotypic resistance to first and second-line injectable drugs by the usage of the Line Probe assay by adopting previously reported studies (Huang et al. 2011).

Impact of Tellurite on the Growth of Mycobacterium tuberculosis

The toxic impact of tellurite on the proliferation and growth of *Mycobacterium tuberculosis* was estimated by the proliferation in the presence of tellurite. As the control set, another test was run without the presence of tellurite. The tellurite was taken in five equal intervals between 1-5 mM respectively (K_2TeO_2; procured from Sigma Aldrich). The inoculum of the test strain was made in such a manner that the turbidity becomes 0.5 McFarland standards through the scrapping of 3-4 colonies of MBT from the Lowenstein-Jensen medium. The resulting culture tubes were thereafter inoculated and have been presented in Figure 1. Mycobacterium exhibited proliferation and growth at a concentration of 1 mM and a complete absence of growth was observed within the concentration range of 2-5 nm (exhibited in Figure 1) (Lalitha 2004).

Analysis through the Scanning Electron Microscopy (SEM) and Energy Dispersive X-Ray (EDX)

The mycobacterium cells in both the presence and absence of tellurite were further subjected to SEM analysis in order to compare their morphologies. Thereafter the EDX was carried out for the determination of the interaction between the metal and the microorganisms. This was done for the verification of the metal uptake by the *Mycobacterium tuberculosis*. The sample preparation was done by taking about 1 ml of the sample within a microcentrifuge tube. The tube containing the specimen was subjected to centrifugation for 5 minutes at 14000 rpm. The resultant supernatant was discarded and the pellet was thereafter resuspended in 50 µl double distilled deionized water. Thereafter around 30 µl was dispensed within an aluminium foil which was allowed to dry and fix over the aluminium foil. The resulting sample was thereafter observed through a scanning electron microscope (Nova NanoSEM 450). The system was furnished with an Oxford energy dispersive X-ray microanalysis system (Patil 2016).

RNA Extraction followed by cDNA Synthesis

The extraction of the RNA was conducted by using 1 ml TRIzol which was carried out strictly in accordance with the literature-reported protocol. The colonies were initially scrapped from the Lj culture. Thereafter, they were homogenized in a chilled mortar and pestle along with liquid nitrogen. In the resulting mixture, 1 ml of TRIzol was added (procured from Thermo Scientific, USA). After that, it was treated with chloroform and isopropanol. The resulting pellet was ethanol (70%) washed and resuspended in RNase-free water (40 µl). The extracted RNA was quantified using a Nanodrop plate (Skanit 4.1, Thermoscientific). Subsequently, the mixture was converted into cDNA by using a cDNA synthesis kit (Vivantic cDSK01-050^{TM}) (Yin et al. 2019).

Real-time Polymerase Chain Reaction

The Real-time polymerase chain reaction was carried out using Mic PCR (Bio Molecular Systems^{TM}). The *mmpl7* gene was harnessed for the relevant expression profiling. As the housekeeping gene, the DNA Gyrase A was used. The Real-Time PCR reaction master mix used in this study was the Eva Green qPCR master mix procured from Solis Biodyne, Estonia. The sequence of the primers is indicated below in Table 1. All the experiments were carried out in triplicates and the relative expression was estimated using the double delta C_t method (Xu et al. 2015).

Table 1. The sequence of various primers used in this study

Primers	Sequence (5´-3´)
DNA Gyrase A R	AATGCTTCGCGTATAGC
DNA Gyrase A F	GTCGATTGCCGGATAGCAGGCAT
mmpL R	GTTAAAGCTGCTAGCATAGA
mmpL F	ATGCGCTGATATTCGACTGC

RESULTS

Bacterial Strain Characterization

The bacterial strain used in this study was characterized as XDR-TB through the genotypic resistance up to second-line anti-tuberculosis drugs. The first line of drugs indicated resistance-conferring to the isoniazid (*Kat*G gene), and rifampicin (*rpo*B gene). On the other hand, the second line of anti-tuberculosis drugs conferred resistance to second-line injectables (*rrs* gene) and fluoroquinolones (FQs). A detailed account of the mutations of the genes has been indicated in Table 2.

Table 2 Mutation profiling of the anti-tuberculosis drugs

Gene	Failing wild-type band	Mutant band	Mutation
rss	*rss*WT1	*rss* MUT1	A1401G
gyrA	*gyrA* WT3	*gyrA* MUT3C	D94G
KatG	*KatG* WT	*KatG* MUT1	S315T1
rpoB	*rpoB* WT8	*rpoB* MUT3	S521L

Impact of Tellurite on the Growth of Mycobacterium tuberculosis

A stunned growth of the Mycobacterium in the presence of tellurite was observed as compared to the growth of the control set. This 2 – 3 weeks were observed in both the media (LJ and MGIT). The reduction and uptake of Tellurite by the microorganisms were indicated by the presence of black-coloured colonies. On the contrary, the absence of black colour (the presence of a creamy white-coloured colony) is indicated in the control set of experiments. The bacteria under observation indicated growth only at the concentration of 1 mM. No growth was found within the range of 2-5 mM. The obtained experimental results have been gleaned in Figure 1.

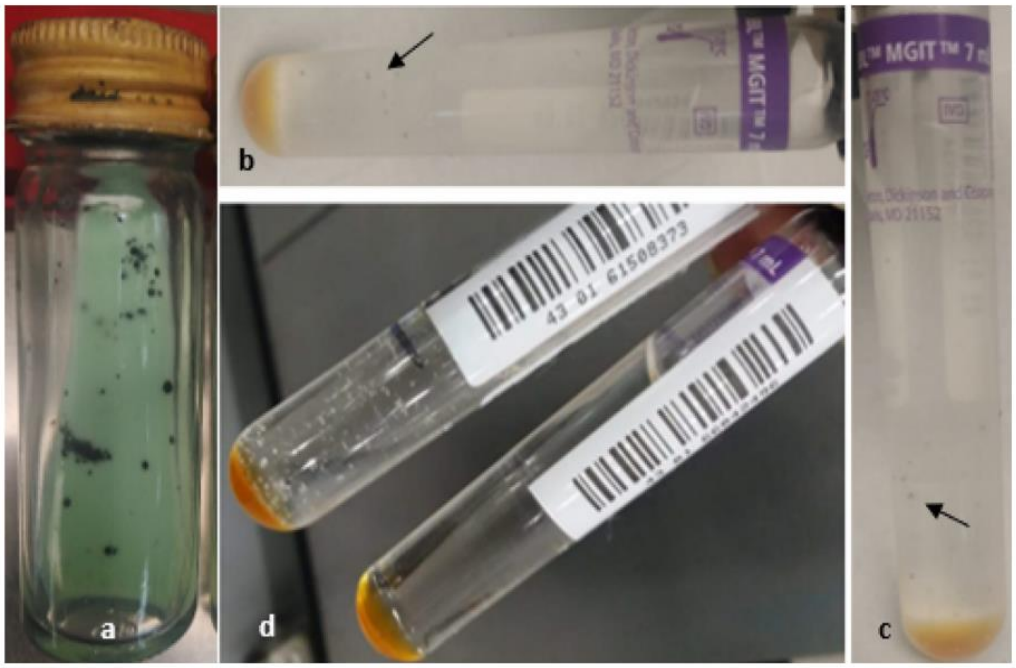

Figure 1 Impact of tellurite on the growth of the microorganisms; (a) black coloured colonies indicating the translation of K_2TeO_3 into black tellurium (Te^0); (b) & (c) MGIT medium indicating minute blackish colonies in the presence of Te; (d) Growth of the microorganisms in the absence of Te (Control specimen) and MGIT control set up which is free from the inoculum.

Analysis through the Scanning Electron Microscopy (SEM) and Energy Dispersive X-Ray (EDX)

The tellurite uptake was confirmed from the EDX analysis. Such a conclusion was arrived at by the signature peak of tellurite within the tellurite-treated cells. This observation has been indicated in Figure 2a. On the contrary, no such peaks were observed in the control experimental set indicated in Figure 2b. The scanning electron microscope analysis indicated a variation in the size of the cells as well as the assemblage of the metallic black tellurite within the cell (Figures 2c, and 2d).

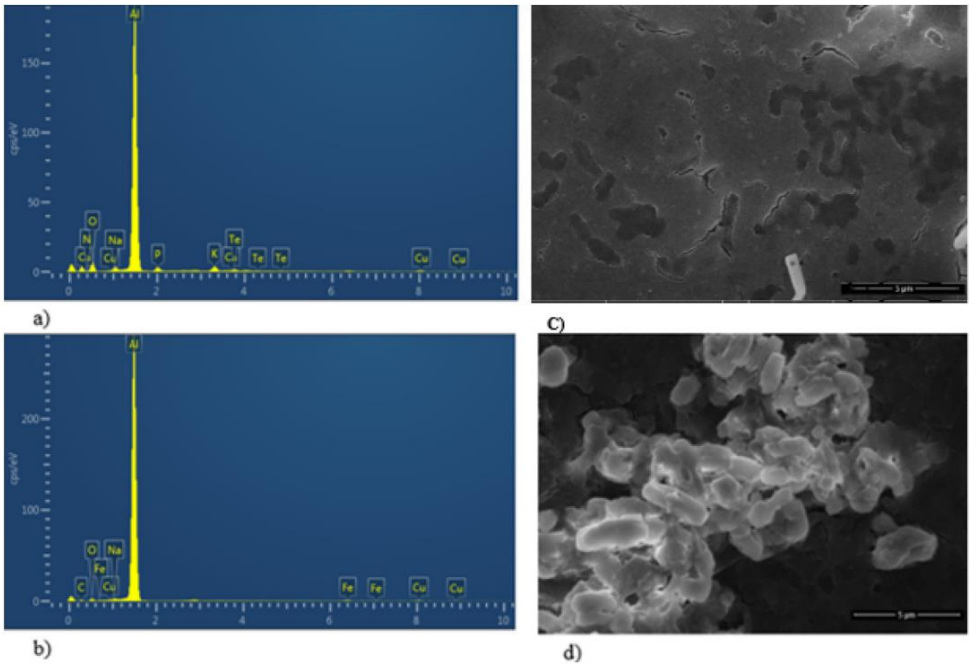

Figure 2 Analysis of the microbial strain through EDX (a) in the presence of tellurite (1mM) which in turn indicated the Te update by the signature peaks; (b) Without the presence of tellurite (c) SEM analysis of the strain with tellurite accumulated within the cell indicating black colour. (d) Scanning Electron Microscopy image of the control specimen in the absence of tellurite.

Relative expression of the drug efflux pump gene

The inhibitory impact of tellurite on the mmpL7 efflux system was assessed through the relative expression of the particular genes in both the absence and presence of Tellurite oxyanions. The analysis of this expression in turn revealed a minor depletion of the efflux pump gene in the presence of tellurite. The obtained results have been indicated in Figure 3.

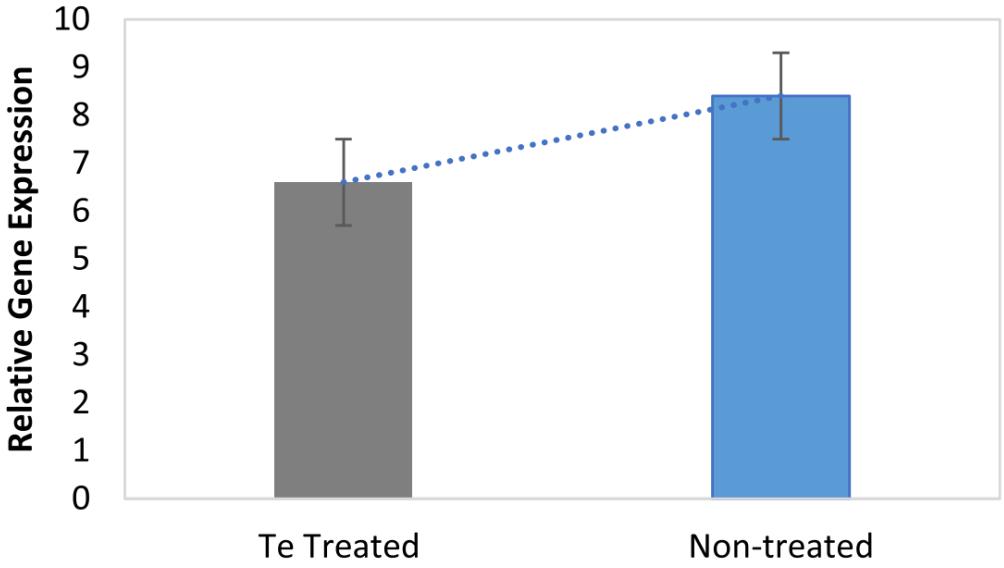

Figure 3 The relative expression of the *mmpL7* gene both in the absence and presence of Tellurite.

DISCUSSION

The previously conducted studies indicated that the K_2TeO_3 has been used as a potential antimicrobial agent (Mason et al. 2023). A spectrum of diseases has been reported to be cured like dermatitis, leprosy, tuberculosis, syphilis etc. by the application of this particular compound at a very low concentration (≤ 5 µM) (Kumar et al. 2021). Moreover, this compound has been reported to have antagonistic efficacy against both prokaryotic as well as eukaryotic

cells. However, the lethal dose against eukaryotic organisms is much higher (reported within the range of 50-1500 µM) as per the various literature reports (Butts et al. 2018).

After an exhaustive literature search, the authors noticed that very few studies related to the antagonistic impact of the tellurite against *Mycobacterium tuberculosis* have been reported. *Mycobacterium tuberculosis* has been reported to acquire resistance against various drugs through genetic mutations. The efflux pump of the drugs has a notable impact on maintaining the drug concentration within the cells which in turn might result in the potential drug resistance within the bacteria (Mishra et al. 2015). Although the use of tellurite to suppress the growth of *Mycobacterium tuberculosis* has been adopted widely, however, the use of tellurite against the inhibition of the XDR-TB growth by inhibiting the efflux pump is the novelty of this current study. Accordingly, in this study, the growth and proliferation of the microorganisms in the presence of tellurite were found to be blackish in appearance. This black colour was due to the reduction of the tellurium which becomes insoluble and thereby forms a black precipitate within the bacterial colonies. The tellurite reduction is not strictly linked to the tellurite resistance since a few tellurite-sensitive strains have also been reported to have tellurite-reducing activity (Lohmeier-Vogel et al. 2004). The observation obtained from this study confirmed the presence of tellurite which in turn has affected significantly inhibited their growth in contrast to the control set of experiment. Such differential growth rate indicates the potential toxicity of the metals which inhibited their growth. Another possibility of the absorption of the metal within the microbial system could be the cationic nature of the metal which also had an impact on their morphology and appearance (Handy et al. 2008). In this study no observation was found in the morphology of the microbes, however, the reduced size of the microbial cells was noticed through the usage of electron microscopy. The obtained results were found to be in sync with previously reported literature reports. The tellurite uptake by the XRD strain of *Mycobacterium tuberculosis* was confirmed by their respective characteristic peak through EDX analysis. Similar reports have been published earlier in the literature for the uptake of cadmium (Maier et al. 2007).

The literature indicated that microorganisms have developed various strategies to combat the intracellular deposition of heavy ions and metals. One such strategy is the use of an efflux pump (Jacob et al. 2018). The efflux pumps of *Mycobacterium tuberculosis* provide resistance against various anti-tuberculosis drugs that enter into the cells. The overexpression of the MmpL7 protein has been found to be associated with high resistance to the first-line anti-tuberculosis drug (isoniazid) where no trace of genetic mutation was observed (Biswas et al. 2021). The drug efflux inhibitors are also found to be very helpful in decreasing efflux-assisted resistance. A lot of studies have been reported to have the activity of various types of inhibitors against the drug efflux pumps such as reserpine, verapamil, thioridazine etc. to name a few (Kaur et al. 2021). In this study, an emphasis was put forward to determine the impact of tellurite on the drug efflux pump. The variation in the expression of the mmpL7 gene under the stress of metal was evaluated. The obtained results gleaned that no potential difference in the expression of the gene was observed in the presence or absence of tellurite stress.

CONCLUSION

In this study, Te was found to have growth supressing potential against the XDR-TB at very minimal concentration (1 mM). XDR-TB displayed blackish colonies in the presence of tellurite and their growth was delayed up to 21 days as compared to their control set. The accumulation of the metal, diminished cell size and morphology along with the signature tellurite peak was found to be present within the metal-contaminated microbial cells. The MBT indicated the MIC value of 1 mM and also displayed high susceptibility for elevated concentrations (2-5 mM). The tellurite concentration was not found to be associated with notable inhibitory efficacy on the mmpL7 efflux system. However, the impact of the metal on the other genes of the efflux system resulting in the inhibition of the growth of microbial growth and proliferation could also be determined and may be considered as one of the potential future scopes of this study.

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