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Genetic analysis of pyrazinamide resistance: mutations in the pncA gene among clinical isolates of multi-drug resistant *Mycobacterium Tuberculosis*

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ABSTRACT

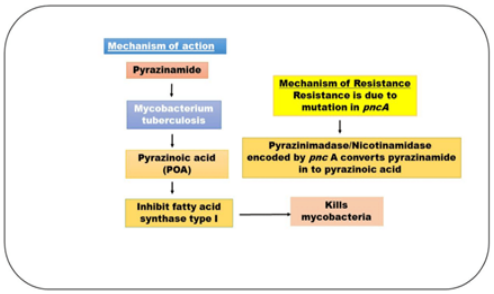
Pyrazinamide (PZA) resistance presents a significant challenge in tuberculosis (TB) management, particularly in multi-drug resistant (MDR) cases. Understanding the genetic basis of PZA resistance is crucial for effective treatment strategies.

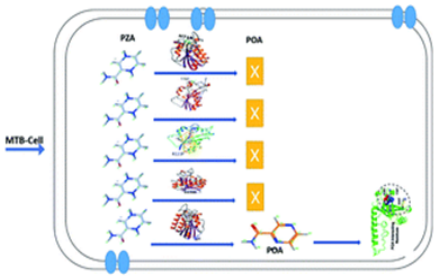
ABSTRACT

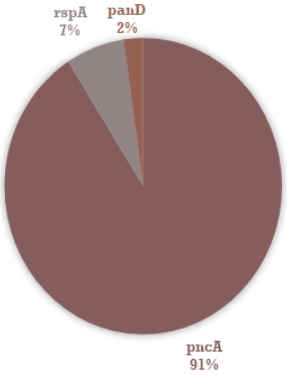
Pyrazinamide (PZA) resistance presents a significant challenge in tuberculosis (TB) management, particularly in multi-drug resistant (MDR) cases. Understanding the genetic basis of PZA resistance is crucial for effective treatment strategies. This review provides a comprehensive overview of recent advancements in molecular characterization of PZA resistance, focusing on mutations in the pncA, rpsA, and panD genes.

STUDY POPULATION & RESULTS

In this study, all 40 clinical isolates investigated exhibited mutations within the pncA gene, distributed across three distinct regions. Remarkably, we identified three isolates with previously unreported mutations at position rspA, and one isolate harboring panD mutations in addition to mutations in pncA. Notably, all 40 clinical isolates displayed alterations in the pncA gene and demonstrated a lack of pyrazinamidase (PZase) activity.







CONCLUSION

These findings have significant implications, particularly in the context of developing rapid tests for detecting PZA-resistant M. tuberculosis strains. Given the observed high frequency of mutations in the pncA gene among PZA-resistant strains, detecting mutations in this gene could serve as a reliable and efficient method for identifying PZA resistance in clinical isolates. This could potentially aid in the timely management and treatment of tuberculosis, allowing for more tailored therapeutic approaches and improved patient outcomes.

This review provides a comprehensive overview of recent advancements in molecular characterization of PZA resistance, focusing on mutations in the pncA, rpsA, and panD genes. We discuss the prevalence of PZA resistance, mechanisms of resistance, and the utility of genetic analysis in early diagnosis. Key findings from studies investigating PZA resistance in clinical isolates are summarized, highlighting the predominant role of pncA mutations. Additionally, we explore the significance of mutations in rpsA and panD genes, elucidating alternative pathways to PZA resistance. Insights from genetic analysis have implications for personalized treatment approaches and TB control programs. Future directions in research and the potential of molecular methods in enhancing PZA susceptibility testing are also discussed.

Keywords: Pyrazinamide, Multi-drug resistant tuberculosis, PncA genes, Mutations.

INTRODUCTION

Pyrazinamide (PZA), an analog of nicotinamide, has been a cornerstone of tuberculosis (TB) treatment for nearly five decades [1]. It serves as one of the important and crucial component among first-line drug anti TB regimens which is being used for short-course chemotherapy against *Mycobacterium tuberculosis*, the causative agent of TB. PZA demonstrates bactericidal activity, particularly against semi-dormant mycobacteria, and plays a pivotal role in reducing the overall duration of TB treatment when used alongside other key drugs such as isoniazid and rifampin. As a result, PZA has gained an utmost importance as a third most vital drug currently being used in modern TB treatment protocols [2]. PZA is assumed to have a unique mode of action in shortening the TB treatment. It has a capability of targeting and eliminating at least 95% of the semi or dormant TB bacilli those which usually persists in lower pH environments. The bactericidal role of the drug is activated in acidic conditions, typically encountered in areas of active inflammation. In simpler terms, PZA's effectiveness lies in its capability to eradicate the majority of dormant bacteria thriving in acidic environments, contributing significantly to the efficiency of TB treatment regimens [3].

Pyrazinamide operates as a prodrug, necessitating activation by a bacterial enzyme called pyrazinamidase (PZase) to transform into its active form, pyrazinoic acid (POA), which exhibits toxicity against *Mycobacterium tuberculosis*. While the precise target of POA within the bacterium remains elusive, it has been proposed that the accumulation of POA triggers a decrease in pH levels. This pH alteration subsequently induces a non-specific inhibitory impact on cellular metabolism. Put simply, POA, once activated, disrupts bacterial functions by causing a decrease in pH, thereby interfering with cellular processes essential for the survival of *M. tuberculosis* [4, 5]. Recent studies have revealed that pyrazinoic acid (POA) has the ability to disrupt the membrane potential of Mycobacterium tuberculosis, thereby impacting transport functions particularly in acidic environments [6, 7]. Pyrazinamide's enzyme which is encoded by pncA gene is not essential for the survival of the bacilli. Mutations occurring in the pncA gene have been extensively studied and are known to confer resistance to pyrazinamide. These mutations can occur throughout the open reading frame of the pncA gene as well as in its regulatory region.

The primary consequence of these mutations is the loss of PZase activity, rendering the mutant incapable of activating pyrazinamide to form POA. In simpler terms, mutations in the pncA gene result in a dysfunctional PZase enzyme, leading to the inability to convert pyrazinamide into its active form, POA, thereby causing resistance to the drug. Conventional methods for testing the susceptibility of Mycobacterium tuberculosis to pyrazinamide, such as agar proportion or Lowenstein–Jensen proportion methods, are labor-intensive and prone to high rates of discordance between different laboratories. This variability can arise due to factors like differences in media pH and other testing parameters. While automated systems like the BACTEC 460 TB and BACTEC MGIT 960 are available commercially which have reduced. Several studies have demonstrated that mutations leading to the inactivation of the pncA gene are the primary mechanism underlying resistance to pyrazinamide [8]. In simpler terms, when the pncA gene is mutated and its function is compromised, the bacterium becomes resistant to pyrazinamide [9]. Hence this current laboratory based prospective study was aimed in determining the rate of PZA resistance among MDR TB specimens that were received in Intermediate Reference Laboratory, Bengaluru and also to study the type of mutations conferring to PZA resistance among these clinical isolates.

METHODOLOGY

Clinical Samples: The study incorporated 1023 sputum samples obtained from individuals suspected of having multidrug-resistant tuberculosis (MDR TB), which were received at the Intermediate Reference Laboratory in Bengaluru. Each specimen underwent processing in accordance with the established standard operating procedures of the laboratory. This meticulous adherence to protocol ensured consistency and reliability in the handling and analysis of the samples.

Sputum Processing for Liquid culture: To certain volume of expectorated sputum samples double the volume of 4% Sodium Hydroxide and the container/ falcon tubes were vortexed or shaken gently for about 1 minute, and then placed on shaker and shaken for around 20 minutes. Later the containers were removed and centrifuged at a rate of 4000rpm for 15 minutes. The supernatant was discarded in appropriate freshly prepared disinfectant as per laboratory SOP and 20ml of sterile water is added and the same step is repeated. Later the sediment is subjected to inoculation onto LJ Media for isolation of

MTB and the culture isolates were further processed for PZA resistance detection using automated BACTEC MGIT 960 systems.

Reagents

MGIT systems was developed by Becton Dickinson and the tube contains an appropriate mixture of modified 7H9 broth with a pH of 5.9 containing Tris 4,7-diphenyl-1,10 phenanthroline ruthenium chloride pentahydrate as fluorescent indicator within a rubber base made of silicone. The PZA resistant kit provided by manufactures usually contains 2 vials of lyophilized PZA and 6 vails of supplements.

Drug Concentration

The final concentration of PZA achieved is 100 μg/ml

Preparation of Inocula for MGIT 960

Before inoculation, 800ul of supplement was added to growth control as well as the PZA tubes provided and 100ul of reconstituted PZA was transferred aseptically using a pipette. Instructions provided by the manufacturer was followed.

Liquid cultures that are positive by MGIT were tested for PZA susceptibility that are either flagged positive from day 1 or not less than 5 days. Undiluted inoculum was used if it is first day of positive flagged and those that are flagged positive from day 3 a 5 fold dilution was made using sterile saline. 500 ul of this diluted inoculum was transferred into the PZA tubes using a sterile pipette. Similarly 500ul of 1:10 diluted suspension was added to the growth control.

Cultures as young as 2 weeks on LJ Media was used for PZA susceptibility testing. Colonies were scrapped with a sterile inoculation loop and a 0.5 Mc Farland suspension was prepared and homogenised using glass beads. Initially 1:5 diluted 500 ul of the suspension was added to these PZA tubes and loaded into MGIT 960 automated systems.

Interpretation of MGIT 960 system

Results of the system were interpreted according to the established criteria for calculating susceptible, resistant, and borderline results as per the instructions manual.

Quality Control

The study utilized *M. tuberculosis* strain H37Rv (ATCC 27294) for each batch of BACTEC MGIT 960 PZA medium and BACTEC MGIT 960 PZA drug. Quality control checks were conducted at least weekly using the specified ATCC strains. In adherence to the study protocol, strict inclusion and exclusion criteria were applied to patients and their clinical specimens. These measures were implemented to ensure the accuracy and reliability of the study's findings.

RESULTS

During the study period spanning from 2019 to 2023, a total of 1023 clinical samples were collected at the Intermediate Reference Laboratory in Bangalore for the diagnosis of multidrug-resistant tuberculosis (MDR TB). Among these samples, 623 (60.89%) originated from male patients, while the remaining 400 (39.10%) were from female patients. This gender distribution provides insight into the demographic characteristics of the individuals seeking diagnosis and treatment for MDR TB during the study timeframe.

Out of the total samples received, 583 (56.98%) were submitted for the diagnosis of drug-resistant tuberculosis (DRTB), while 79 (7.72%) were specifically for the diagnosis of drug-susceptible tuberculosis (DSTB). Additionally, 124 (12.12%) samples were for the follow-up of DRTB cases, and 237 (23.16%) were for the follow-up of DSTB cases. These figures delineate the distribution of samples based on their intended diagnostic and follow-up purposes, offering insights into the priorities and focus areas of tuberculosis management during the study period.

During the study period in Karnataka, PZA resistance was identified in 40 out of 1023 patients (3.9%) through liquid culture testing. Among these PZA-resistant patients, 20 (50%) were previously diagnosed with pulmonary tuberculosis (PTB) and were receiving anti-tubercular treatment (ATT) when their samples were sent for drug-resistant tuberculosis (DRTB) diagnosis, as per guidelines. Additionally, 11 patients (26.8%) were known cases of DRTB and were already undergoing DRTB treatment according to guidelines. Both these groups had a history of ATT intake. The remaining 9 patients (21.95%) without any prior history of ATT intake were newly diagnosed cases whose samples were sent for drug-susceptible tuberculosis (DSTB) diagnosis.

Among the total PZA-resistant patients, 23 (56.09%) were male and 18 (43.90%) were female. The overall prevalence of PZA resistance observed in this study in Karnataka during the study period was 4%. These findings shed light on the distribution of PZA resistance among different patient groups and provide insights into the epidemiology of drug-resistant tuberculosis in the region.

All 40 samples that showed resistance to pyrazinamide (PZA) through liquid drug susceptibility testing (DST) were cultured on Lowenstein-Jensen (LJ) media to isolate the Mycobacterium tuberculosis (MTB) pathogens. Remarkably, all 40 samples resulted in the growth of MTB isolates. Subsequently, DNA extraction was performed on these isolates, followed by amplification of the DNA and purification of the polymerase chain reaction (PCR) products, all conducted according to the specified protocol outlined in the methodology section. Finally, the purified PCR products were sequenced to analyze the genetic characteristics of the MTB strains present in the samples.

MUTATIONS AMONG PZA RESISTANT ISOLATES

Out of the 40 pyrazinamide (PZA) resistant Mycobacterium tuberculosis (MTB) isolates that underwent sequencing, mutations were detected in various genes associated with PZA resistance. Specifically, 37 isolates (92.5%) exhibited mutations in the pncA gene,

with 97.27% of these mutations being single nucleotide substitutions and 2.70% resulting in frame shift mutations.

Additionally, 3 isolates (8.10%) demonstrated mutations in both the pncA and rpsA genes, while 1 isolate (2.5%) displayed mutations in both the pncA and panD genes. These findings provide insights into the genetic basis of PZA resistance in MTB strains, highlighting the predominance of mutations in the pncA gene and the occurrence of mutations in other genes associated with PZA resistance (Table 1)

Table 1: showing mutations conferred to pncA, rpsA and panD genes among PZA resistant MTB isolates.

pncA mutations					
Isolate number	**SL.NO**	**Nucleotide position**	**Nucleotide change**	**Amino acid position**	**Amino acid change**
M_01	1	85	T to C	29	Leu to Pro
M_02	2	minus 12	T to C	na	Promoter
M_03	3	138	G to A	46	Cys to Tyr
M_04	4	145	G to A	49	Asp to Asn
M_05	5	85	T to C	29	Leu to Pro
M_06	6	286	A to C	96	Lys to Gln
M_07	7	139	G to C	47	Val to Leu
M_08	8	359	T to G	120	Leu to Arg
M_09	9	85	T to C	29	Leu to Pro
M_10	10	391	G insertion	131	Frameshift mutation
M_11	11	minus 12	T to C	na	Promoter
M_12	12	329	A deletion	110	Frameshift mutation
M_13	13	132	TC insertion	44	Gly to Ser
M_14	14	286	A to C	96	Lys to Gln
M_15	15	215	G to C	72	Cys to Ser
M_16	16	145	G to A	49	Asp to Asn
M_17	17	56	T to C	19	Leu to Pro
M_18	18	286	A to C	96	Lys to Gln
M_19	19	171	G to C	57	Ala to Pro
M_20	20	215	G to C	72	Cys to Ser
M_21	21	391	G insertion	131	Frameshift mutation
M_22	22	56	T to C	19	Leu to Pro
M_23	23	minus 12	T to C	na	Promoter
M_24	24	286	A to C	96	Lys to Gln
M_25	25	85	T to C	29	Leu to Pro
M_26	26	137	C to T	46	Ala to Val
M_27	27	511	G to A	171	Ala to Thr
M_28	28	85	T to C	29	Leu to Pro
M_29	29	215	G to C	72	Cys to Ser
M_30	30	145	G to A	49	Asp to Asn
M_31	31	56	T to C	19	Leu to Pro
M_32	32	minus 12	T to C	na	Promoter
M_33	33	56	T to C	19	Leu to Pro
M_34	34	145	G to A	49	Asp to Asn
M_35	35	85	T to C	29	Leu to Pro
M_36	36	85	T to C	29	Leu to Pro
M_37	37	56	T to C	19	Leu to Pro
M_38	38	139	G to C	47	Val to Leu
M_39	39	minus 12	T to C	na	Promoter
M_40	40	359	T to G	120	Leu to Arg
rpsA mutations					
M_06	1	532	A to G	178	Lys to Glu
M_14	2	636	A to C	212	Arg to Arg
M_23	3	636	A to C	212	Arg to Arg
panD mutation					
M_08	1	167	T to C	56	Val to Ala

Discussion & Conclusion

This paper aims to elucidate the global challenge posed by the emergence and dissemination of multi drug-resistant (MDR) Mycobacterium tuberculosis strains, which significantly impede tuberculosis (TB) control efforts. Specifically, the focus lies on delineating the progress made in comprehending the molecular mechanisms underlying M. tuberculosis resistance to pyrazinamide (PZA), and exploring the potential of molecular techniques in promptly diagnosing PZA-resistant TB. In this study, all 40 clinical isolates investigated exhibited mutations within the pncA gene, distributed across three distinct regions. Remarkably, we identified three isolates with previously unreported mutations at position rspA, and one isolate harboring panD mutations in addition to mutations in pncA. Notably, all 40 clinical isolates displayed alterations in the pncA gene and demonstrated a lack of pyrazinamidase (PZase) activity.

Our findings underscore the prevalence of pncA gene mutations in PZA-resistant M. tuberculosis strains, underscoring the potential for developing a rapid diagnostic test to identify PZA-resistant M. tuberculosis strains. This research sheds light on novel insights into the

molecular basis of PZA resistance, which can inform the development of effective diagnostic strategies and aid in combating the spread of drug-resistant TB.

In this study, the importance of the PncA, rpsA, and panD genes lies in their association with pyrazinamide resistance in Mycobacterium tuberculosis. Specifically:

PncA gene

The PncA gene encodes pyrazinamidase, which is crucial for activating pyrazinamide, an essential drug used in tuberculosis treatment. Mutations in the PncA gene can result in reduced or absent pyrazinamidase activity, leading to resistance against pyrazinamide. Since the majority of pyrazinamide resistance in M. tuberculosis is attributed to mutations in the PncA gene, analyzing this gene can provide valuable insights into the resistance mechanisms and guide treatment strategies.

RpsA gene

Mutations in the rpsA gene have also been linked to pyrazinamide resistance in M. tuberculosis. The rpsA gene encodes the ribosomal protein S1, and alterations in this gene may affect the expression or function of the PncA enzyme, thus influencing pyrazinamide susceptibility. Studying mutations in the rpsA gene alongside PncA can enhance our understanding of the genetic basis of pyrazinamide resistance.

PanD gene

The panD gene is implicated in pyrazinamide resistance, although the precise mechanism is not fully elucidated. This gene encodes aspartate decarboxylase, involved in pantothenate biosynthesis. Mutations in panD may disrupt metabolic pathways or cellular processes, contributing to pyrazinamide resistance. Investigating the role of panD mutations in resistance mechanisms can provide comprehensive insights into the genetic determinants of pyrazinamide resistance.

Pyrazinamide-resistant strains with diverse mutations in the pncA gene do not display any loss of fitness or virulence. Isolates lacking alteration in the pncA gene are reported to have mutations in the rpsA (ribosomal protein I) gene. Overexpression of rpsA has also been implicated in increased resistance to pyrazinamide. However, there was no clear demonstration that mutations in rpsA were linked to pyrazinamide resistance. A small proportion of resistant isolates lack mutations in the pncA gene, suggesting another mechanism of resistance exists. More recently, panD mutations have been associated with pyrazinamide resistance. WGS analysis revealed the presence of panD mutations in pyrazinamide-resistant isolates and the inclusion of these in screening has been recommended to enhance the detection of pyrazinamide resistance.

In this study, the findings indicate a strong correlation between mutations in the pncA gene and the lack of pyrazinamidase (PZase) activity, suggesting that alterations in the pncA gene are a predominant mechanism of pyrazinamide (PZA) resistance in Mycobacterium tuberculosis. Specifically:

Mutations in pncA

All 40 clinical isolates examined in the study displayed mutations in the pncA gene. This indicates that mutations in pncA are highly prevalent among PZA-resistant M. tuberculosis strains.

Localization of mutations

The mutations in the pncA gene were found to be localized to three specific regions of the gene. This suggests that certain regions of the pncA gene are more prone to mutations associated with PZA resistance.

Results of this study have significant implications, particularly in the context of developing rapid tests for detecting PZA-resistant M. tuberculosis strains. Given the observed high frequency of mutations in the pncA gene among PZA-resistant strains, detecting mutations in this gene could serve as a reliable and efficient method for identifying PZA resistance in clinical isolates. This could potentially aid in the timely management and treatment of tuberculosis, allowing for more tailored therapeutic approaches and improved patient outcomes. Overall, by examining mutations in the PncA, rpsA, and panD genes, this study aims to establish correlations between genetic variations and pyrazinamide resistance in *M. tuberculosis*, facilitating more effective diagnosis and treatment strategies for tuberculosis patients.

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