Assessing the significance of the β-actin gene in tuberculosis diagnosis: an analytical study utilizing anal fistula specimens

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Abstract

Fistula samples from suspected tuberculosis (TB) patients play a crucial role in diagnosing advanced extrapulmonary TB. This study examined 70 clinical specimens of anal fistulae suspected of Mycobacterium tuberculosis (MTB) infection using both conventional and molecular methods. The β-actin gene, a housekeeping gene, was assessed for TB detection while microbiological profiling identified acid-fast bacilli. Molecular profiling yielded positive results for both the IS6110 and β-actin genes in 14 samples, suggesting MTB infection. In 13 samples, only the β-actin gene was positive, indicating inflammation caused by other pathogens. Bayesian analysis was conducted to evaluate the diagnostic performance of both gene tests.

The IS6110 gene test showed a sensitivity of 95% and specificity of 98% with a positive predictive value (PPV) of 0.819 and a negative predictive value (NPV) of 0.993. The β-actin gene test demonstrated a sensitivity of 90%, specificity of 99%, PPV of 0.966 and NPV of 0.984. Bayesian calculations indicated a posterior probability of 0.791 for TB in specimens positive for both genes. The findings emphasize the β-actin gene as a potential marker for TB infection. Its detection in TB-negative samples suggests the presence of alternative pathogenic microorganisms. This discovery could be crucial for confirming diagnoses, particularly when radiological methods are unable to identify pathogens.

Keywords: Tuberculosis, Fistulae, Housekeeping gene, β-actin gene.

Introduction

Fistulas, which are abnormal connections between organs or tissues, often arise in advanced or untreated cases of extrapulmonary tuberculosis (EPTB)[24]. Specimens for analysis are usually obtained through minimally invasive methods such as aspiration or biopsy, providing valuable material for various diagnostic assessments including microbiological cultures, molecular assays and histopathological examinations.[25] Given the diagnostic hurdles associated with TB, particularly in extrapulmonary instances, these samples offer a direct route to identify the presence of MTB, the causative agent of TB, facilitating prompt diagnosis and treatment commencement[26,27].

Housekeeping genes, which are genes perpetually active in fundamental cellular functions, assume a pivotal role as reference points for gene expression scrutiny in biological specimens. In the realm of TB diagnosis using fistula samples, housekeeping genes are instrumental in ensuring the precision and dependability of molecular diagnostic methods such as polymerase chain reaction (PCR) assays[16,20,27,48]. By gauging the expression levels of target genes linked to TB such as those encoding specific TB antigens or virulence factors, relative to housekeeping gene expression, researchers can discern the presence of MTB in fistula samples with heightened accuracy. This standardization procedure helps to mitigate disparities in sample quality, RNA integrity and experimental conditions, thereby augmenting the sensitivity and specificity of TB detection techniques[42].

Furthermore, housekeeping genes are essential for interpreting gene expression data from fistula samples, as they provide a baseline for comparative analysis across different patient specimens or experimental conditions. By quantitatively assessing the expression levels of housekeeping genes, researchers can evaluate how various factors such as disease severity, host immune response and treatment effectiveness affect the molecular profile of tuberculosis (TB) in these samples. This comprehensive approach not only supports TB diagnosis but also offers insights into disease progression, host-pathogen interactions and treatment outcomes, thereby paving the way for personalized treatment strategies and improved patient care[27,48].

Material and Methods

EPTB affects organs beyond the lungs, encompassing areas such as the pleura, lymph nodes, abdomen, genitourinary tract, skin, joints, bones and meninges. Bacteriologically confirmed cases denote instances where MTB growth is observed through culture. Clinically diagnosed cases rely on robust PCR techniques[17,24,35,42]. Anal fistulas, particularly complex ones, are well-known for their high recurrence rates and resistance to treatment. There are various causes responsible for non-healing and recurrences of fistulas and

inability to detect TB is one of them. If a coexisting TB infection in the fistula goes undetected, then the chances of fistula healing after surgery are quite low. Either the fistula will not heal or there would be a recurrence after few weeks to months of surgery[28,31,34,39,51].

There are numerous diagnostic methods in use to identify TB in anal fistulas. The most common and one of the oldest methods is histopathological examination (HPE). Several new tests have also been developed in last three decades but most of them have fallen out of favor. The only tests which demonstrate better detection rate than HPE, are polymerase chain-reactions (PCR). Bayesian statistical analysis was utilized for the diagnostic uncertainty.

Study subjects: From February 2024 to April 2024, clinical specimens of anal fistulae suspected of MTB were sourced from multiple hospitals and nursing homes and were examined at DNA Labs - A Centre for Applied Sciences, Dehradun.

Ethical approval: The Institutional Ethics Committee granted approval for the study under reference number SIMS 131/IEC-SKIMS/2016-146, dated February 2024.

Eligibility criteria: Individuals of all ages and genders exhibiting compelling clinical or radiological signs of tuberculosis, along with a prescription for AFB smear and TB PCR were included.

Exclusion criterion: Confirmed cases of pulmonary tuberculosis in patients undergoing antitubercular treatment at the time were excluded from participation in the study. Additionally, samples displaying contamination in cultures or molecular analyses were also excluded.

Inclusion criterion: The study comprised samples (n=70) for which results were accessible from all conventional methods including ZN staining and TB PCR techniques.

Study groups: The sample set (n=70) constituted the extrapulmonary tuberculosis group, characterized by positive TB PCR results obtained from anal fistula samples.

Sample processing: Each sample underwent both conventional and molecular methods for the detection of *M. tuberculosis*. Extrapulmonary samples, particularly fistulae, were collected by the treating physician under stringent aseptic conditions and then dispatched to the laboratory.

Pre-processing of Fistulae samples: Sample processing was performed within a Class II B2 Biosafety Cabinet. Samples obtained from "sterile" sites were concentrated by centrifugation while others underwent decontamination using Petroff's method with 4% NaOH. Anal fistula biopsies were initially ground in 4 mL of saline before decontamination. All samples were then centrifuged at 13,000 rpm for 10 minutes at 4°C. The supernatant was carefully separated and the remaining sediment was stored at -20°C for the detection of MTB using multiplex PCR to confirm positive results.

Additionally, two to three milliliters of sediment obtained after concentration or decontamination were used to prepare smears for Ziehl-Neelsen (ZN) staining. Following the confirmation of TB-PCR results, the remaining sediments were processed to confirm the presence of the β-actin gene and the IS6110 gene using conventional and gradient PCR methods.

Microbiological profiling: The microbiological profiling of MTB involved the Ziehl-Neelsen (ZN) acid-fast staining method, a specialized technique for detecting acid-fast bacilli including MTB in clinical samples. Initially, a small amount of the clinical specimen from fistula biopsies was evenly spread onto a glass slide to create a smear, ensuring uniform distribution for subsequent staining. Heat fixation was then applied to the smear by either passing it through a flame or using a hot air oven, which facilitated the adherence of the sample to the slide and prevented it from washing off during the staining process. The smear was then flooded with carbol fuchsin, a primary stain containing basic fuchsin and phenol, followed by gentle heating to enhance the penetration of the stain into the mycobacterial cell wall.

Rinsing with acid-alcohol or an acidified alcohol solution removed the primary stain from non-acid-fast organisms while retaining it in acid-fast bacteria like MTB. A counterstain, such as methylene blue or brilliant green, was then applied to visualize non-acid-fast organisms, providing contrast against the acid-fast bacilli. The stained smear was examined under a light microscope using oil immersion at high magnification, typically 1000x. Acid-fast bacilli appeared as bright red or pink rods against a blue or green background, while non-acid-fast organisms appeared blue or green.

DNA Isolation: The spin column method was used for nucleic acid purification, starting with the lysis of biological samples to release nucleic acids. This was followed by the binding of the nucleic acids to a silica-based matrix within the spin column under optimized buffer conditions. After loading the sample onto the column, centrifugation separated the nucleic acids bound to the matrix from contaminants, allowing the flow-through to be discarded. Subsequent washing steps removed residual impurities, ensuring high purity of the nucleic acids. Elution of the purified DNA from suspected TB was achieved by disrupting the interactions between the nucleic acids and the silica matrix, releasing them into a collection tube for downstream applications, primarily PCR.

Amplification by PCR: PCR standardization for the β-actin gene required primer selection which involved the design and synthesis of oligonucleotide primers as well as sequencing.

PCR Programing Standardization for β- actin gene: Post-amplification, the detection of amplified products (amplicons) was conducted via agarose gel electrophoresis (AGE) where agarose gel, 0.5 g of agarose was added to 40 ml of 1X TAE buffer.

Results

Microbiological Profiling results: Following Ziehl-Neelsen (ZN) acid-fast staining, the presence and quantity of acid-fast bacilli including MTB were noted in clinical specimen, with the number per high-power field (HPF) reported semi-quantitatively as 1+, 2+, 3+, or 4+, indicating increasing bacterial load as mentioned in table 4.

Cellular and Molecular Profiling: DNA from all fistula samples was analyzed using conventional PCR in a Biorad endpoint thermal cycler, resulting in amplicons of 123 base pairs for the IS6110 gene and 306 base pairs for the β-actin gene. The PCR technique confirmed the presence of mycobacterial genomes in the specimens from patients (Table 4). Among the 70 specimens analyzed, 14 tested positive for both the IS6110 and β-actin genes. Additionally, 8 specimens were positive only for the IS6110 gene, while 13 specimens were positive only for the β-actin gene. Participants represented a range of age groups, with a mean age of 39.36 years. There were 38 male cases, making up the largest group and 32 female cases.

Table 1
ligonucleotide primers for β- action Gene

Primers	Gene Sequence (5' to 3')	Size(bp)
β-actin Forward Primer	TCACCCACACACTGTGCCCATCTACGA	306
β -actin Reverse Primer	CAGCGGAACCGCTCATTGCCAATGG	

Table 2
Reagents preparation for the First step for standardization of β-actin gene PCR Amplification

S.N.	Components	Volume for 1 rxn
1.	PCR Buffer	5µl
2.	dNTPs	5µl
3.	$Mgcl_2$	5µl
4.	β- actin Primer $_{1\ forward}$	0.5µl
5.	β- actin Primer $_{2\ reverse}$	0.5µl
6.	Taq DNA polymerase.	0.5µl
7.	NFW	9 µl
Total		**30 µl**
DNA Template		**10 µl**

Table 3
Polymerase Chain Reaction cycling conditions in End Point Thermal Cycler

S.N.	Stages of PCR	Temperature	Time	No. of cycles
1.	Initial Denaturation {First Hold}	94°C	05 minutes	1
2.	Denaturation	94°C	30 seconds	41X
3.	Annealing	59°C	30 seconds	
4.	Extension	72°C	30 seconds	
5.	Final extension {Second Hold}	72°C	05minutes	1
6.	Storage/Incubation	4°C	∞{Infinity}	

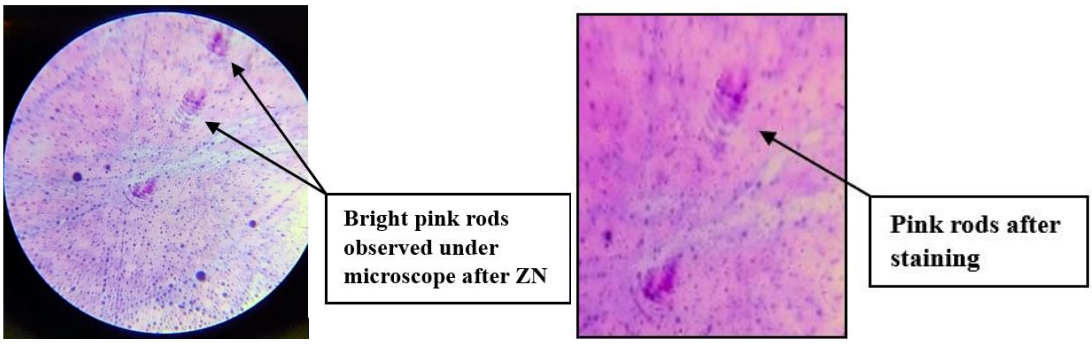

Figure 1: Detection of acid-fast bacilli, including *Mycobacterium tuberculosis* (MTB) by ZN Staining under microscope

Table 4
Data depicting the different gene targets, staining and age/gender wise distribution

Case No.	Age in years	Gender (M/F)	IS6110 Gene	β-actin gene	Acid-fast bacilli (AFB) present	Quantity of AFB per high-power field (HPF)	Appearance of Acid-Fast Bacilli (AFB)
1	24	M	Positive	Positive	Yes	2+	Bright pink rods
2	28	M	Negative	Negative	No	-	-
3	16	F	Negative	Negative	No	-	-
4	63	M	Positive	Positive	Yes	3+	Bright pink rods
5	64	F	Negative	Negative	No	-	-
6	77	M	Positive	Positive	Yes	4+	-
7	29	F	Negative	Positive	No	-	-
8	38	F	Positive	Positive	Yes	2+	Bright Red rods
9	24	F	Positive	Negative	No	-	-
10	36	M	Negative	Positive	Yes	1+	Bright Red rods
11	18	F	Positive	Positive	Yes	3+	Bright Red rods
12	26	M	Positive	Negative	Yes	2+	Bright pink rods
13	34	M	Negative	Negative	No	-	-
14	52	F	Positive	Positive	Yes	2+	Bright Red rods
15	64	M	Negative	Negative	No	-	-
16	53	F	Negative	Positive	No	-	-
17	38	F	Negative	Positive	No	-	-
18	27	F	Positive	Negative	Yes	3+	Bright Red rods
19	13	M	Negative	Negative	No	-	-
20	47	M	Negative	Negative	No	-	-
21	29	M	Positive	Positive	Yes	1+	Bright Red rods
22	76	M	Negative	Negative	No	-	-
23	40	M	Negative	Positive	No	-	-
24	73	F	Negative	Negative	No	-	-
25	33	M	Positive	Positive	Yes	2+	Bright pink rods
26	61	F	Negative	Negative	No	-	-
27	29	F	Negative	Negative	No	-	-
28	43	M	Positive	Positive	Yes	2+	Bright Red rods
29	27	M	Negative	Negative	No	-	-
30	22	F	Positive	Negative	Yes	3+	Bright Red rods
31	54	M	Negative	Positive	No	-	-
32	31	M	Negative	Negative	No	-	-
33	26	F	Negative	Positive	No	-	-
34	23	M	Negative	Positive	No	-	-
35	14	F	Negative	Negative	No	-	-
36	24	F	Negative	Negative	No	-	-
37	16	M	Positive	Negative	Yes	1+	Bright pink rods
38	69	M	Negative	Negative	No	-	-
39	18	M	Negative	Negative	No	-	-
40	39	F	Negative	Negative	No	-	-
41	20	F	Positive	Positive	Yes	2+	Bright Red rods
42	43	M	Negative	Negative	No	-	-
43	22	M	Negative	Negative	No	-	-
44	56	M	Negative	Positive	No	-	-
45	67	M	Positive	Negative	Yes	4+	Bright pink rods
46	55	F	Negative	Negative	No	-	-

47	43	M	Positive	Negative	Yes	1+	Bright Red rods
48	38	M	Negative	Negative	No	-	-
49	28	M	Negative	Positive	No	-	-
50	33	M	Negative	Negative	No	-	-
51	28	F	Negative	Negative	No	-	-
52	38	F	Positive	Negative	Yes	2+	Bright pink rods
53	41	F	Positive	Positive	Yes	2+	Bright pink rods
54	46	M	Negative	Negative	No	-	-
55	72	F	Negative	Negative	No	-	-
56	25	M	Negative	Negative	No	-	
57	88	F	Negative	Positive	No	-	-
58	31	F	Negative	Negative	No	-	-
59	20	F	Positive	Positive	Yes	3+	Bright pink rods
60	13	M	Negative	Negative	No	-	-
61	74	F	Negative	Negative	No	-	-
62	35	M	Negative	Positive	No	-	-
63	27	F	Negative	Negative	No	-	-
64	21	F	Negative	Negative	No	-	-
65	83	M	Positive	Positive	Yes	4+	Bright pink rods
66	54	F	Negative	Negative	No	-	-
67	20	M	Negative	Positive	No	-	-
68	58	M	Negative	Negative	No	-	-
69	37	M	Negative	Negative	No	-	-
70	21	M	Positive	Positive	Yes	2+	Bright pink rods
Mean Age	39.36 Years						

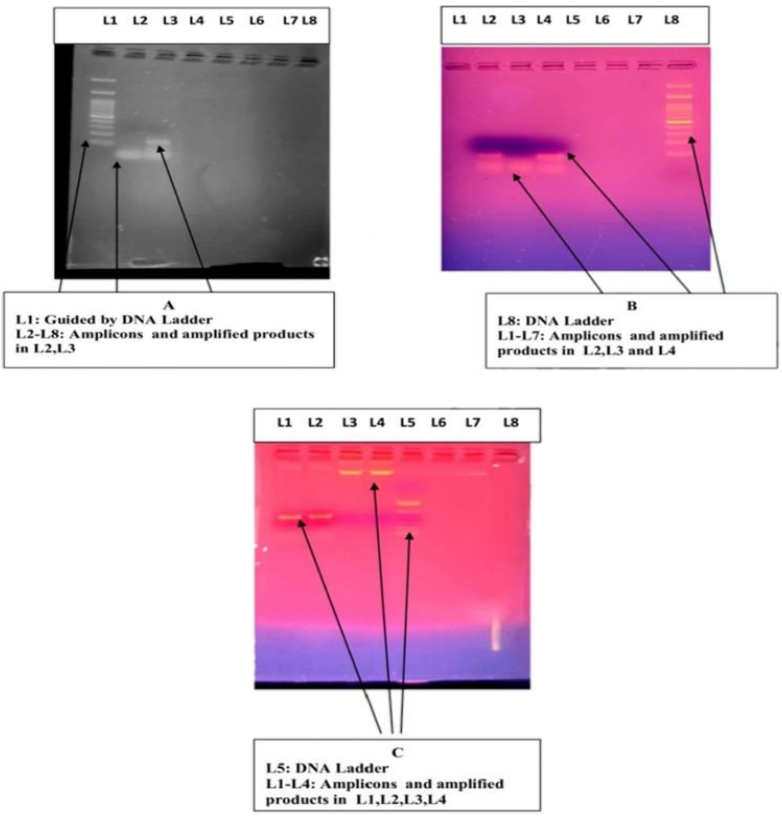

Figure 2: Gel Picture Showing Band for β-actin gene
Figure A displays a 306 base pair amplicon size for the β-actin gene in wells L2 and L3.
Figure B illustrates a 306 base pair amplicon size for the β-actin gene in wells L2, L3 and L4.
Figure C presents a 306 base pair amplicon size for the β-actin gene in well L1, L2 and L5.

The β-actin gene holds significant importance in the realm of diagnosing TB due to its potential as a crucial marker. This gene serves as an invaluable indicator of inflammation triggered by bacterial infections, offering insights beyond the identification of MTB alone. Notably, cases that yield negative results for the IS6110 gene, a commonly used target for TB detection, yet test positive for the β-actin gene, point towards inflammation caused by organisms other than MTB. These organisms encompass a range of bacterial pathogens, including *Streptococcus pneumoniae*, *Listeria monocytogenes*, *Haemophilus influenzae* and *Streptococcus pneumoniae*[15,32,41,46]. This distinction is essential in clinical settings where TB symptoms are present, but traditional diagnostic methods do not confirm MTB infection.

In such cases, detecting the β-actin gene offers valuable information about the presence of other pathogenic microorganisms contributing to the observed inflammation. By differentiating between TB and other bacterial infections, the β-actin gene helps clinicians to make accurate diagnoses and implement appropriate treatment strategies tailored to the specific pathogen responsible for the inflammatory response. Based on the provided data, several observations and interpretations can be made regarding the diagnosis and management of TB, focusing on the presence of the IS6110 gene, the β-actin gene, the presence and quantity of acid-fast bacilli (AFB) per high-power field (HPF) and the appearance of AFB. From table 4, it can be observed that the presence of the IS6110 gene, specific to the MTB complex, is noted in several cases, which is essential for confirming MTB infection.

Additionally, the β-actin gene, used as a control, is positive in instances where the IS6110 gene is present, further validating the reliability of the PCR assay. Furthermore, acid-fast bacilli (AFB) are observed in several cases, with quantities per high-power field (HPF) varying from 1+ to 4+, indicating different levels of bacterial load. The appearance of AFB also varies, described as bright pink or bright red rods. The presence of both the IS6110 gene and AFB confirms active tuberculosis infection in these cases. The variation in AFB quantity and appearance may indicate different stages of infection and potentially varying responses to treatment. Cases where AFB is absent despite the presence of the IS6110 gene might suggest early or controlled infection, or may reflect limitations in sampling or testing sensitivity. The mean age of 39.36 years indicates a diverse age range affected by tuberculosis, aligning with the general epidemiological profile of the disease.

Based on the data from figures A, B and C where a 306 base pair (bp) amplicon of the β-actin gene was consistently detected across multiple wells, while the IS6110 gene specific to MTB complex was negative, several considerations and implications can be discussed as the consistent detection of the 306 bp β-actin gene amplicon in figures A (wells L2 and L3), B (wells L2, L3 and L4) and C (wells L1, L2 and L5). PCR assays were performed reliably and reproducibly across different experimental setups. This consistency is essential in molecular diagnostics, ensuring that the amplification process is robust and the DNA extracted from the samples is suitable for analysis. The absence of the IS6110 gene, which is specific to MTB complex, despite the presence of the β-actin gene amplicon, suggests several important implications for disease diagnosis.

Applying Bayesian analysis, prior probabilities obtained for having TB was P(TB) 0.3143 and probability of not having TB was P(Not TB) 0.6857. Sensitivity of IS6110 gene test (Se(IS6110 | TB)) was 95%, specificity of IS6110 gene test (Sp(IS6110 | Not TB)) was 98%, sensitivity of β-actin gene test (Se(β-actin | TB)) was 90% and specificity of β-actin gene test (Sp(β-actin | Not TB)) was 99%. Considering the values, IS6110 gene test positive predictive value obtained was ≈ 0.819 and negative predictive value (NPV(IS6110)) was 0.993. Likelihood ratio for IS6110 (LR(IS6110)) was 47.5. For β-actin gene test PPV(β-actin) was 0.966, NPV (β-actin) was ≈ 0.984 and LR (β-actin) was 90. Using Bayes' theorem, posterior probabilities P(TB | IS6110+β-actin+) for TB were calculated for specimens positive for both IS6110 and β-actin genes (14) specimens and the value obtained was ≈0.791.

Given P(IS6110+β−actin+|TB) ≈0.791, the probability P(TB|IS6110+β−actin+)P(TB|IS6110+β-actin+)P(TB|IS61 10+β−actin+) is calculated considering the joint effect of both tests' PPVs and the prevalence of TB in the population. This calculation yields a posterior probability that helps clinicians interpret the likelihood that a patient with positive results for both genes actually has TB. The study evaluated 70 clinical specimens, focusing on the presence of both IS6110 and β-actin genes. Among these specimens, 14 tested positive for both genes, indicating a combined molecular signature highly suggestive of TB. The Bayesian analysis allowed us to calculate posterior probabilities, emphasizing the importance of the joint detection of these genes in enhancing diagnostic precision.

The high positive predictive values (PPV) for both IS6110 (0.819) and β-actin (0.966) genes reinforce their utility in confirming TB diagnosis when both are detected. Clinically, the results provide valuable guidance for healthcare practitioners in confirming TB cases accurately. The likelihood ratios (LR) for IS6110 (47.5) and β-actin (90) genes underscore their respective roles in TB detection, with higher LR indicating stronger diagnostic capability. This information aids in clinical decision-making, particularly when determining the need for initiating TB treatment based on molecular testing outcomes.

The study's assumptions on test sensitivity and specificity (95% and 98% for IS6110; 90% and 99% for β-actin) provide a theoretical basis for the Bayesian calculations. These parameters highlight the importance of robust test characteristics in optimizing diagnostic accuracy.

Real-world application would necessitate validation of these parameters in the specific population under study. To analyze the possibility of other pathogens when the β-actin gene is positive but TB is not detected, total number of specimens were (n)70, specimens positive only for β-actin gene (not IS6110 gene) were (n)13 where (P(TB)) = 0.3143 (based on specimens positive for IS6110 gene) and (P(Not TB)) = 1 - P(TB) = 0.6857, (Se(β-actin | TB)) = 90% and (Sp(β-actin | Not TB)) = 99%, The positive predictive value (PPV) for β-actin gene test when TB is not present, was approx. 0.988.

The PPV(β-actin | Not TB) is approximately 0.988, meaning that if the β-actin gene is positive but TB is not detected (based on IS6110 gene negativity), there is a high probability (98.8%) that the positive β-actin result is accurate and indicative of another pathogen. Negative predictive value (NPV) for β-actin gene test when TB is not present was 0.957. The NPV (β-actin | Not TB) is approximately 0.957, indicating a 95.7% probability that a negative β-actin result correctly indicates the absence of TB. These Bayesian calculations demonstrate that a positive β-actin gene result, in the absence of TB as indicated by negative IS6110 gene results, is highly likely to be accurate (PPV = 98.8%). This suggests that the specimen may contain DNA from another pathogen that shares sequence similarity with the β-actin gene primer used in the PCR assay.

Conversely, a negative β-actin gene result is highly indicative (NPV = 95.7%) of the absence of TB, supporting the reliability of this gene as a control and its specificity in TB diagnostics. This analysis reinforces the importance of the β-actin gene as a reliable control in TB diagnostics. Its high specificity ensures accurate detection of TB-negative cases, thereby guiding clinicians to consider alternative pathogens in patients with clinical symptoms resembling TB but negative TB molecular tests. This approach aids in comprehensive patient management and ensures appropriate treatment strategies based on accurate diagnostic outcomes.

Discussion

Fistulae TB, alternatively known as tuberculous fistulae or tuberculous sinus, arises as a complication of TB infection which is caused by the bacterium MTB. While TB primarily affects the lungs, it can also target other body areas like lymph nodes, bones, joints and skin. Fistulae tuberculosis develops as TB infection advances, forming channels or tunnels that link the affected internal organ to the skin surface[38,43,52,53]. These channels may appear in various body regions including the lungs, abdomen and skin. In cases of cutaneous tuberculosis affecting the skin, fistulae TB typically manifests as draining sinuses or ulcers on the skin surface[30,31,37,52]. These sinuses often discharge purulent material, potentially containing TB bacteria and can be painful, persisting if not adequately treated.

The emergence of fistulae TB signifies an advanced TB stage and may relate to factors like delayed diagnosis, inadequate treatment, or drug resistance[21,22,29,30,36,37]. Treatment typically involves a combination of antibiotics effective against MTB, occasionally complemented by surgical intervention to remove infected tissue or repair affected organs. Early diagnosis and prompt treatment initiation are vital in halting TB progression and averting complications such as fistulae[15,28,40]. Moreover, public health measures such as TB screening, contact tracing and ensuring treatment adherence play pivotal roles in curbing TB spread and reducing complications like fistulae TB[34,40,45,51,52].

The research aimed to explore the significance of the β-actin gene in fistulae for the cellular and molecular characterization of MTB. β-actin, a highly conserved protein expressed in all eukaryotic cells, serves as a stable cytoskeletal protein and a loading control in PCR. The study involved analyzing clinical samples from patients in Dehradun exhibiting symptoms like fever, headache, vomiting, cough etc. Out of 70 samples processed, those showing positive results for β-actin gene PCR were more likely associated with MTB infections. This suggests that β-actin gene could serve as a significant marker for MTB infections, aiding in differentiation from other inflammatory conditions. Notably, the presence of β-actin gene was also detected in patients with fistulae infections not linked to MTB, implying its potential utility in distinguishing between TB and other inflammations caused by various pathogens.

The identification of β-actin gene in patients negative for tuberculosis indicates the presence of other pathogenic microorganisms triggering inflammation[10-12,18,19,41]. This finding could be particularly beneficial in confirming diagnoses when TB symptoms are present but radiological methods fail to detect pathogens such as in tuberculosis meningitis cases. Based on the provided data and Bayesian analysis, several key implications and considerations emerge regarding the detection of the β-actin gene in the context of TB diagnosis and the potential presence of other pathogens. The consistent detection of the 306 bp β-actin gene amplicon across multiple experimental setups (Figures A, B and C) indicates robust and reliable PCR assays. This consistency is crucial in molecular diagnostics, ensuring that the amplification process is dependable and the DNA extracted from specimens is suitable for analysis.

Importantly, the absence of the IS6110 gene, specific to the MTB complex, alongside the presence of the β-actin gene amplicon, raises significant implications for disease diagnosis. The Bayesian analysis provided posterior probabilities (P(TB | IS6110+β-actin+)) for TB based on joint detection of both IS6110 and β-actin genes. With 14 specimens testing positive for both genes, the calculated posterior probability of TB was approximately 0.791.

This emphasizes the utility of combining these molecular markers to enhance diagnostic precision, especially when both genes are detected concurrently. The study reported

high PPVs for both IS6110 (approximately 0.819) and β-actin (approximately 0.966) genes, indicating their effectiveness in confirming TB diagnosis when positive.

Conversely, high NPVs for these tests (approximately 0.993 for IS6110 and 0.984 for β-actin) suggest their reliability in ruling out TB when negative. These values are essential for clinicians to accurately interpret test results and make informed decisions regarding patient management. When the β-actin gene is positive but the IS6110 gene is negative (in 13 specimens), Bayesian calculations indicate a high positive predictive value (PPV) of approximately 0.988 and a negative predictive value (NPV) of about 0.957. This suggests that a positive β-actin result in the absence of TB is likely to indicate the presence of another pathogen that shares sequence similarity with the β-actin gene primer used in the PCR assay.

Conversely, a negative β-actin result strongly indicates the absence of TB, reinforcing its specificity as a control gene in TB diagnostics. These findings highlight the β-actin gene's reliability as a control in TB diagnostics, ensuring accurate detection of TB-negative cases and prompting consideration of alternative pathogens in patients with clinical symptoms resembling TB but negative molecular test results. This approach supports comprehensive patient management, allowing clinicians to tailor treatment strategies based on precise diagnostic outcomes.

The thorough Bayesian analysis and molecular diagnostic findings emphasize the importance of utilizing multiple genetic markers in TB diagnosis. The robustness of the PCR assays, along with the high predictive values of the IS6110 and β-actin genes, enhances diagnostic accuracy and clinical decision-making, ultimately improving patient care. Further validation of these findings in diverse patient populations would strengthen their applicability in real-world settings, underscoring the ongoing evolution and refinement of TB diagnostic strategies.

Conclusion

This study underscores the pivotal role of the β-actin gene in TB diagnosis at both cellular and molecular levels. The β-actin gene, a fundamental marker in this study, served not only as a control gene but also as a critical indicator of inflammation associated with bacterial infections beyond MTB. The findings reveal that while the IS6110 gene is specific to MTB and confirms active TB infection in cases where it was detected, the β-actin gene's presence in specimens negative for IS6110 highlights alternative pathogenic microorganisms contributing to inflammatory responses. These findings are particularly significant in clinical settings where traditional TB diagnostic methods fail to detect MTB but clinical symptoms persist, suggesting the presence of other bacterial pathogens.

Therefore, the β-actin gene's detection aids clinicians in accurately diagnosing TB and differentiating it from similar presentations caused by other pathogens, thereby guiding appropriate treatment strategies. Moreover, the study utilized Bayesian analysis to assess the diagnostic accuracy of combining IS6110 and β-actin gene detections. The analysis yielded high positive predictive values (PPV) for both genes when detected together, emphasizing their complementary roles in confirming TB diagnosis with confidence.

Conversely, when the β-actin gene was detected but IS6110 was absent, the high PPV indicated that the specimen likely contained DNA from other pathogenic microorganisms, distinct from MTB. This reinforces the β-actin gene's specificity in detecting TB-negative cases and prompts consideration of alternative pathogens in clinical management.

Furthermore, the study's validation of PCR assays across different experimental setups underscores the reliability of these molecular diagnostic tools in clinical practice, ensuring robust and reproducible detection of genetic markers crucial for TB diagnosis[34].

Fistulae tuberculosis is a serious infection associated with significant mortality and high morbidity among the survivors. The only way to reduce mortality and morbidity is by early diagnosis and timely recognition of complications following the progression of the disease and in post-treatment follow-up[2,12,33,45]. A correlation between disease severity and PCR positivity could be clinically useful but, given the variability in the clinical manifestation of fistulae, TB and other infections need to be evaluated further. The above protocol for β-actin gene can be further standardized by multiplexing with IS6110 and mbp64 genes for the diagnosis and validation of Internal control for MTB complex[1,3,4,6,7,47].

Future research should continue to explore the β-actin gene's utility across diverse patient populations to further validate its role in refining TB diagnostic protocols and enhancing global efforts towards TB control and eradication. The cases which were positive for IS6110 gene and negative for β-actin gene, there can be a possibility of latent TB which can be further studied by interferon gamma release assays etc., differentiating between latent and active TB[5,7-9,13,14,30,36,37].

Furthermore, ongoing global efforts toward TB eradication require continued research into the genetic diversity of MTB strains and their interactions with human hosts. This research will facilitate the development of personalized diagnostic approaches tailored to regional strain variations, ultimately optimizing treatment outcomes and reducing the risk of drug resistance.

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