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Review

Molecular Viability Assay: Improving Leprosy Diagnosis beyond Current Gold Standard

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Abstract. Neglected tropical diseases are still part of the health problems faced by the world. One of the neglected tropical diseases that has not yet reached 100% elimination is leprosy. *Mycobacterium leprae* is the pathogen responsible for leprosy, a chronic infectious disease that affects the skin and peripheral nerves and can lead to significant disability if left untreated. Currently, the gold standard for diagnosis is detecting acid-fast bacilli (AFB) with Ziehl-Neelsen staining; however, this method cannot distinguish between living and dead bacteria, complicating treatment assessment, relapse detection, and resistance tracking. Therefore, more accurate diagnostic instruments that can differentiate bacterial viability are needed. Since *M.leprae* cannot be cultured in artificial media, molecular-based assays are promising tools for rapid diagnosis. This study aims to identify recent assays for assessing bacterial viability in leprosy. Articles used are limited to the publication year between 2019 until 2024 from databases such as PubMed, ProQuest, Scopus and Google Scholar, using PRISMA methods. After filtration, from 143 articles we found 5 articles that discussed the viability of leprosy-causing bacteria. The selected studies showed that molecular assays to determine bacterial viability can be used and explored to strengthen the existing gold standard for monitoring treatment of leprosy patients

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1. Introduction

Leprosy, as a neglected tropical disease, has been recognized around the world, even since colonial times in Indonesia. Given that the disease's term appears in several religious texts, leprosy can be classified as an "ancient" condition [1-3]. Despite being an ancient disease, new leprosy cases continue to emerge, especially in Indonesia [2],[4]. The prevalence ratio of newly discovered leprosy cases has been trending downward in Indonesia during the last eleven years, but it has risen after 2022 [4]. Indonesia ranks third globally in leprosy cases, alongside Brazil and India [5]. Low- and middle-income developing nations often have leprosy cases and have not yet eradicated the disease [5]. At the national level in 2023, there was a discovery of 14.376 new cases of leprosy in Indonesia, with the three highest provinces being East Java, West Java and Central Java [4]. 1.201 cases of pediatric leprosy were also found in the Indonesian community [4]. The identification of new leprosy cases, specifically in children from year to year within a region, shows that leprosy is actively spreading throughout the community [6-8].

Leprosy is a chronic, contagious skin disease that, if left untreated, can lead to disability in the sufferer caused by *Mycobacterium leprae* [9]. This bacteria is an obligate intracellular, acid-resistant, rod-shaped bacilli and cannot be cultured in vitro on an artificial medium like other bacteria [10]. The incubation period of *M. leprae* can reach 2-20 years, and can only live in living cells [11-12]. In addition to humans, several studies have that *M.leprae* can be zoonotic in some animals, such as armadillos, squirrels and monkeys [9],[13]. To conduct research using these animals is certainly not the best option in leprosy because the incubation time can be quite long compared to incubation time, which is quite long when compared to *M. tuberculosis* [12],[14-16]. These special characteristics cause some challenges in developing leprosy diagnostics and treatment.

The gold standard approach to support the diagnosis of leprosy is a positive test result for *M. leprae* with microscopic acid-fast bacilli-based examination utilizing the Ziehl-Neelsen method on patient skin scrapings known as slit-skin smears [17-19]. Because this analysis is unable to differentiate between living and dead bacteria, it cannot be used for conclusive diagnosis or treatment success assessment (15). Furthermore, in individuals with pausi bacillary (PB) leprosy, microscopic analysis may produce false-negative results due to the extremely low quantity of pathogenic bacteria [15]. Cross-diagnosis with other *Mycobacterium* genus bacteria is also possible due to their comparable shape [10], [14-15].

Numerous molecular approaches have been applied to leprosy diagnostics, advancing our understanding of the disease from multiple perspectives. Conventional PCR and qPCR assays are among the most widely developed techniques for detecting *M.leprae* [20-21]. Sequence-based typing has validated HLA-B*13:01 as a biomarker for Dapsone Hypersensitivity Syndrome (DHS) in leprosy patients, enhancing patient safety by predicting adverse drug reactions [22-25]. Genes associated with drug resistance, such as *folP1*, *rpoB*, and *gyrA,* have also been investigated through molecular assays, providing insights into resistance mechanisms and supporting more effective leprosy treatment strategies [26-28].

The gold standard for examining bacterial viability in leprosy is to use an animal model, known as the Shepard assay using mouse footpad [14],[16]. Although it is the gold standard assay, Shepard assay is time consuming, requires a complex laboratory and it is not easy to maintain animal models. Molecular methods, particularly conventional PCR and qPCR have improved detection sensitivity; however, they cannot differentiate between live and dead bacteria if the PCR test only detects the DNA. The Molecular Viability Assay (MVA) represents a significant advancement by targeting specific RNA transcripts that are only present in live, metabolically active cells. MVA thus offers enhanced diagnostic accuracy and faster turnaround compared to traditional methods [10], [15]. Despite its potential, the widespread application of MVA remains limited due to high costs, inadequate infrastructure in some regions, and the need for trainned personnel.

This review discusses recent advancements in MVA and its potential to improve leprosy diagnosis and treatment monitoring, especially in endemic areas. This review presents the most recent insights on molecular assays for leprosy, aimed at enhancing diagnostic accuracy, particularly in differentiating between viable and non-viable bacteria. A comprehensive understanding of molecular testing's role in identifying *M.leprae* is anticipated to address traditional methods' shortcomings and advance leprosy's control and elimination.

2. Experimental Section

The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement of the 2020 method centred on molecular viability assay in leprosy was used to conduct this systematic review (29). The articles used in this study are published from 2019 until 2024 from various databases such as PubMed, ProQuest, Scopus and Google Scholar. We implemented a search method using the strategy of "Mycobacterium leprae" AND "molecular viability" and excluded any duplicate articles. The following were the requirements for inclusion: (a) studies published between 2019 and 2024, (b) explaining leprosy and viability assays, and (c) describing molecular techniques in viability leprosy analysis. This review article's exclusion criteria were as follows: (1) the study was a narrative review with an abstract but no full script, (2) the studies were published in languages other than English and Indonesian (3) not addressing bacterial viability.

After importing data from several databases into Mendeley reference management software, all articles were consolidated into a single folder. We then thoroughly eliminated duplicate articles to ensure data accuracy. The initial screening step involved reviewing abstracts and titles against our predetermined inclusion and exclusion criteria to discard papers irrelevant to the study's goals. Articles were excluded if only the abstract was available without the full text. Article quality was assessed using modified components of the Newcastle-Ottawa Scale (NOS). This involved evaluating the study design, assay reproducibility, sample size, and clarity of the viability outcome. Each study received a maximum possible score of 9 points. A summary of the NOS evaluation for the included studies is provided below:

Table 1. Quality assessment using the modified Newcastle-Ottawa scale Newcastle-Ottawa Scale (NOS)

Study	Selection (max 4)	Comparability (max 2)	Outcome (max 3)	Total Score (max 9)
Beissner et al. (2019)	★★★	★	★★	6
Turankar et al. (2022)	★★★	★	★★	6
Neumann et al. (2022)	★★★	★	★★	6
Lenz et al. (2022)	★★★★	★★	★★★	9
Collins et al. (2023)	★★★★	★★	★★★	9

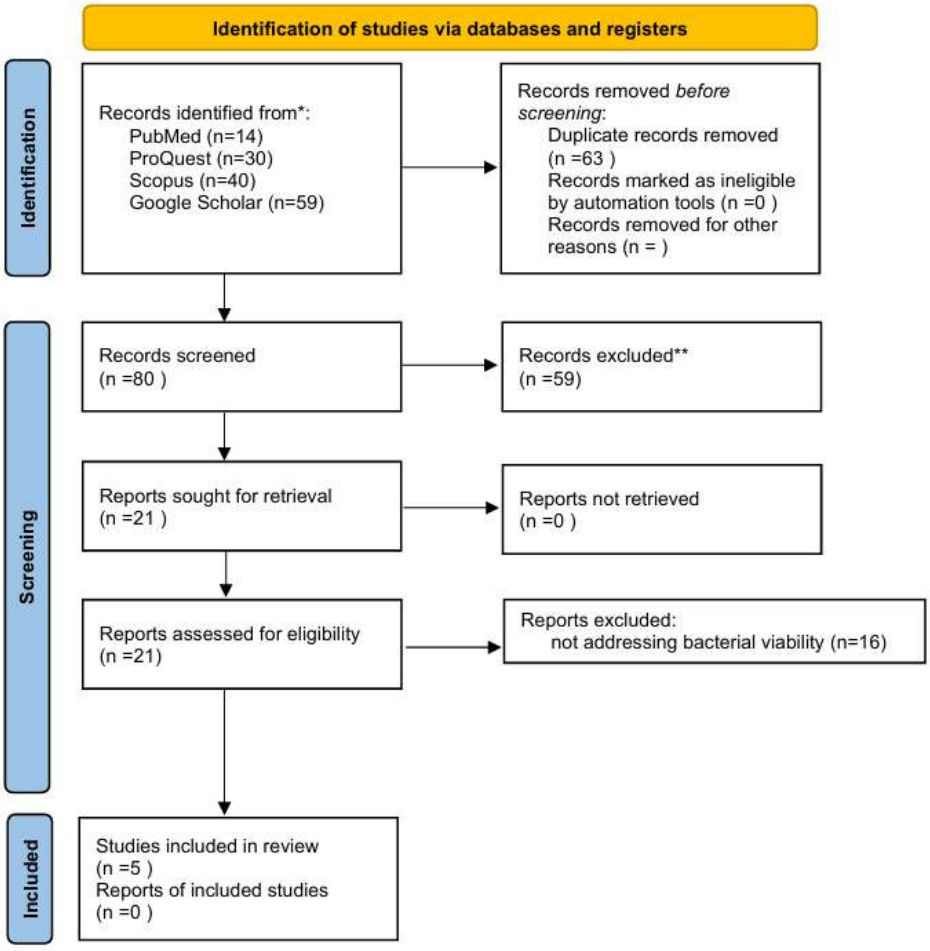

Figure 1. Flow of the study selection using PRISMA

Figure 1 depicts this thorough selection procedure using the PRISMA flow diagram. A comprehensive search across all databases using the specified keywords yielded 143 articles. Additionally, 63 duplicate articles were eliminated. We reviewed the research publications and any relevant reviews in the acknowledged reports. The abstracts of titles that might qualify were screened. As a result of their failure to meet the inclusion and exclusion criteria, 59 of the 80 items eligible for consideration were rejected. Due to the absence of bacterial viability assessment methods, these studies were excluded from the articles selected for review. A selection of five papers meeting the inclusion criteria was considered eligible for assessment

3. Results and Discussion

3.1. Leprosy

The etiological agent of leprosy is *Mycobacterium leprae*, an obligate intracellular bacterium [12]. This slow-growing microorganism is Gram-positive, possesses a rod morphology (measuring 1−8 µm in length and 0.3−0.5 µm in diameter), is non-motile, and is resistant to acids and alcohol [12],[30]. A defining characteristic of *M. leprae* is its inability to be cultured on artificial media [10]. It replicates via binary division, a notably slow process that takes approximately 12−14 days at 27∘C [12],[19]. This protracted division rate contributes to an extremely long incubation period, which can range from 2

to 50 years [15],[31-32]. The bacterium's thick cell wall features an outermost capsule composed of phenolic glycolipids (PGLs), along with other essential components such as peptidoglycan, arabinogalactan, mycolic acid, phthiocerol dimycocerosate (PDIM), lipoarabinomannan (LAM), and lipomannan (LM) [12],[30]. Interestingly, the bacterial load in a leprosy patient's body does not always correlate directly with the severity of clinical symptoms [30],[33].

The World Health Organization (WHO) divides leprosy into two main categories: MB (Multi Bacillary) and PB (Pausi Bacillary) [19],[30]. The main difference between these two groups is the number of lesions on the patient's body [11]. PB patients are those patients with five or fewer skin lesions, while MB patients are those with more skin lesions. WHO classification is widely used in health services in Indonesia [5],[11],[34].

3.2. Gold Standard for Viability Assay in Leprosy: The Shepard Assay

The primary approach for leprosy diagnosis is through clinical examination and histopathologic examination, which specifically identifies acid-resistant bacilli (BTA) in skin or nerve biopsies [19-20]. This method allows direct microscopic visualization of *M.leprae* in tissue samples using Ziehl Nelssen stain [10],[19]. Although this test is the gold standard for leprosy, it cannot distinguish between live and dead bacteria, so it cannot be used to determine bacterial viability [33]. This makes the test unusable for definitive diagnosis and accurate evaluation of treatment success [14],[33]. In addition, microscopic examination may give false negative results in leprosy patients, related to the number of bacteria. Due to similar morphology, there is also the possibility of cross-diagnosis with other Mycobacterium genus bacteria.

The mouse footpad (MFP) assay, which involves inoculating mice's footpads with the relevant *M. leprae* bacilli and measuring bacterial growth over many months, is the gold standard for assessing *M. leprae* viability [14],[16]. This assay was developed in 1960 by Charles Shepard and entails injecting a suspension of *M. leprae* into the footpads of immunocompromised mice (usually Swiss albino mice or athymic nude mice) because these animals offer a favorable environment for the slowly growing bacteria without developing a serious illness [16]. Because of its lower temperature and other mycobacteria had been successfully cultivated there, Shepard selected the footpad as the infection site. The bacilli grew with a doubling time of around 13 days when 10^4 *M. leprae* were implanted into the footpad of immunocompetent mice [16]. Despite being reasonably sensitive, this process can impede quick clinical applications, requires comprehensive laboratory and up to a year of culture before data collecting [33]. Leprosy viability can also be assessed in chimpanzees, red squirrels, and nine-banded armadillos (*Dasypus novemcinctus*) [10],[15-16],[35].

Figure 2. Overview of Shepard Assay
Source: Lenz, et al [16] created with Biorender.com

3.3. Molecular Viability Assay: Current Development

Advancements in molecular biology are providing essential tools to overcome challenges in leprosy diagnosis. One of the advances in leprosy diagnosis that has begun to be explored is molecular viability testing based on qPCR. The purpose of this assay is to determine the viability of the bacteria so that it can be further utilized for monitoring leprosy treatment. Several gene targets for developing leprosy molecular viability assays have been explored.

Table 2. Characteristics studies included in review

Author (year)	Subject	Molecular Technique Used	Main Finding
Beissner, et al (2019)	20 MB leprosy patients in Togo	two real-time PCRs that detect *M. leprae* DNA (RLEP qPCR) and RNA (16S rRNA RT qPCR) by grouping pre-treatment samples to "must detect" RLEP/16S rRNA (DNA)" samples and "must not detect" samples	• *M.leprae* was detected in nasal swab samples • Great specificity and sensitivity using the combined RLEP and 16S rRNA RT qPCR assay for determining the bacterial load and viability of *M. leprae*
Turankar, et al (2022)	• Environtmental samples: 1,060 soil samples and 620 water samples from areas where leprosy patients resided • Slit-skin smears: 112 patients	qPCR targeting 16S rRNA gene and RLEP conserved gene areas. • VNTR (variable nucleotide tandem repeat) analysis and SNP (single nucleotide polymorphism) typing to characterize the strains genetically.	• 16SrRNA detected in 44% soil samples and 56% water samples • The detection of viable *M. leprae* in the environment, along with SNP Type 1 *M. leprae* found in both patients and environmental samples, suggests that both environmental reservoirs and infected individuals contribute to leprosy transmission.
Arthur da Silva Neumann et al (2022)	10 MB Patients in Brazil	qPCR analysis of 16S rRNA to assess *M.leprae* viability in nasal and oral mucosa samples	• Found heterogeneity in viability of *M.leprae* even after several months of treatment.
Lenz, et al (2022)	Leprosy patients from 3 countries: the Philippines (n=199) Nepal (n=200) Ethiopia (n=40)	*M. leprae* count using RLEP qPCR on the DNA fraction, Reverse-transcription (RT-PCR) of normalized RNA to generate cDNA, viability assessment utilizing esxA qPCR and hsp18	• Viable *M. leprae* was indicated by the three countries's differing hsp18 and esxA transcript expression rates • MVA can be utilized to track *M. leprae* mortality in patients after medication treatment.
J Hunter Collins, et al (2023)	footpad tissues from immunocompetent BALB/c mice that were infected with a high dose of *Mycobacterium leprae, strain Thai-53*	MVA by utilizing RLEP qPCR on the DNA fraction, viability assessment using hsp18 -esxA qPCR and Mouse Footpad Assay, counted by the method of Shepard & McRae	The MVA demonstrated good sensitivity and specificity in identifying live bacteria, validated by the assay's consistency with the conventional mouse footpad (MFP) assay. *hsp18* and *esxA* were found to be more dependable markers than 16S rRNA because *M.leprae* populations that were determined to be dead by the MFP test showed 16S transcripts

3.3.1. RLEP and 16S rRNA

Beissner et al in 2019 used RLEP and 16SrRNA as targets to validate two real-time PCR assays for detecting viable *M.leprae* from nasal swab samples [32] "Repeated element of Mycobacterium leprae," or RLEP for short, is a particular specific DNA sequence that is employed as a target in molecular assays to identify *M.leprae* [32],[36]. It is crucial for the diagnosis of leprosy and is used in real-time PCR assays to detect the presence of the bacterium in clinical samples, like nose swabs [32],[37]. The specificity of the assays for viable bacteria is supported by the use of molecular viability assays that

target ribosomal RNA (16SrRNA), which are only detectable from viable or replicating bacteria (32,37). This approach ensures that the assays can differentiate between live and dead bacteria, as rRNA is typically present in metabolically active cells. Therefore, the detection of rRNA indicates the presence of viable *M.leprae*, while its absence suggests that the bacteria are not viable [32],[37].

Besides using clinical specimens, the viability of *M.leprae* have also been studied from the water and soil of leprosy patients's living areas. Turankar, et al (2022) [1] targeting the 16SrRNA gene and RLEP-conserved gene regions using real-time PCR (RT-PCR). They successfully detected viable *M. leprae* in 44% of soil samples and 40% of water samples collected from areas where leprosy patients resided. In order to determine the genetic diversity of the natural organism, molecular genotyping of *M. leprae* from patients and the environment using SNP type and/or VNTR analysis was also carried out. This could help in tracking and understanding leprosy transmission. The conclusion that the *M.leprae* found in the samples were alive and connected to locations where leprosy patients lived is further supported by the fact that *M. leprae* was not detected in control areas. These methods are effective because the RNA and specific gene regions are stable and have a slower degradation rate, making them reliable markers for assessing the viability of the bacteria in environmental samples [1].

3.3.2. *hsp18* and *esxA*

The specificity of rRNA for living bacteria is a matter of some question [33]. Because it is found in metabolically active cells, rRNA is typically seen as a sign of viability; however, some research indicates that rRNA can also be found in dead bacteria or metabolically dormant yet culture-negative bacteria. This suggests that rRNA detection might not always indicate the presence of living bacteria because, in some circumstances, it can also originate from non-living cells [14],[15],[32].

Certain mRNA transcripts, *hsp18* and *esxA*, have also been investigated as *Mycobacterium leprae* viability markers [14-15],[38-39]. The production of the protein *hsp18*, which is involved in the bacterial stress response, is a sign of *M. leprae* viability [14],[39-40]. When *M. leprae* grows intracellularly inside macrophages, this protein is precisely controlled at the post-translational level, signalling the proliferation of living bacteria [14],[33],[38]. Another protein linked to *M. leprae's* virulence and survival is *esxA*, whose expression is correlated with the viability of the bacterium [33]. The virulence factor 10 ESAT6, which is encoded by the esxA gene, causes cell-mediated immune responses and the generation of IFN-γ throughout the course of leprosy [33]. Both markers are useful for determining the viability of *M. leprae* in tissues because their expression levels dramatically drop in non-viable bacteria [14],[15],[31].

To verify the Molecular Viability Assay's precision and efficacy, J.Hunter Collins, et al, 2023 compared MVA assay to the MFP assay, the gold standard for assessing *M. leprae* viability using qPCR [14]. The study developed and validated a molecular viability assay (MVA) to assess the viability of *M.leprae* in infected tissues by measuring the normalized expression of specific transcripts (*hsp18*, *esxA*, and 16SrRNA) [10],[14]. The MVA correlated well with the MFP assay, confirming its accuracy as a rapid and sensitive method for determining *M. leprae* viability, which is crucial for monitoring leprosy treatment and managing associated complications [14]. They established cut-off values for these transcripts using receiver operating characteristic (ROC) analyses, demonstrating high sensitivity and specificity in distinguishing viable from non-viable bacteria [14]. Results indicated that while 16S rRNA transcripts were present even in non-viable samples, *hsp18* and *esxA* transcripts served as more reliable viability indicators [14]. The analysis established a clear functional dichotomy between the mRNA transcripts (*hsp18* and *esxA*) and the ribosomal RNA (*16S rRNA*), confirming that *hsp18* and *esxA* are superior indicators of viability when compared against the gold-standard MFP assay.

Table 3. *hsp18 & esxA* and 16S rRNA

Molecular Target	Assessment Role	Advantages	Limitations
hsp18 & esxA	Primary Viability Indicator	High Discriminatory Power (*AUC* up to 0.9997): Precisely distinguishes high-viability from low-viability populations. Growth Correlation: Expression levels associated with bacterial growth observed in the MFP assay. Reliable Exclusion: Established cut-offs enable the MVA to reliably identify dead *M. leprae* (resulting in undetectable expression).	Transcript abundance is naturally lower than ribosomal RNA, requiring higher sensitivity in detection.
16S rRNA	Internal Control (Extraction Quality)	High Abundance: Excellent as an internal control to confirm successful *M. leprae* RNA extraction, even in samples containing dead bacilli.	Unreliable viability indicator: detection does not conclusively indicate viable bacteria. Long term stability: persists in substantial numbers even when bacilli are confirmed dead by MFP at late time points, introducing a significant risk of misinterpreting dead *M. leprae* as viable.

The *hsp18* and *esxA* genes provide a precise and reliable molecular measurement of *M. leprae* viability. These targets are reliable because their mRNA transcripts break down quickly when the bacteria die, allowing them to accurately reflect the live, metabolically active population. In contrast, the 16S rRNA is unreliable for determining viability. It is too stable and persists long after the bacteria are non-viable, leading to false-positive viability readings. Substantial amounts of 16S rRNA are still detectable even 12 months post-infection, when the bacilli have been confirmed dead (showing no growth) based on the MFP test. Therefore, 16S rRNA should only be used to confirm the successful quality of the RNA extraction from the specimen, not to conclude that the *M. leprae* are viable.

Although MVA shows a strong correlation with the gold standard Mouse Footpad (MFP) assay, it is important to recognize potential limitations and risks of error, particularly related to the nature of the molecular targets used. Relying on 16S rRNA without normalization and strict viability thresholds can lead to misinterpretation of dead *M. leprae* as viable. This is a serious limitation that must be avoided in determining drug efficacy. MVA overcomes this limitation by focusing on *hsp18* and *esxA*, which serve as rapidly degradable markers of metabolic activity in dead (non-viable) bacteria.

3.3.3. MVA Performance Across Three Endemic Regions

Exploring these gene targets also have been conducted in three endemic-leprosy countries, Nepal, the Philippines and Ethiopia, by Linda B Adams, et al (2022) [15]. The study found that transcript expression rates differed across new leprosy cases from three different countries, and that both *hsp18* and *esxA* were detectable in their samples across various cohorts [15]. As the duration of multidrug therapy (MDT) grew, they showed that the MVA dramatically decreased the viability of *M. leprae*,

indicating that it can effectively track bacterial death following treatment [15]. This is significant because the long-term survival of dead bacilli in tissues may make clinical assessments of medication resistance and treatment compliance more challenging [15]. The study found a combination of consistency in overall detection but variability in specific transcriptional profiles across the three endemic sites.

Tabel. Endemic sites

Area of Comparison	Finding	Philippines (CSC)	Ethiopia (AHRI)	Nepal (AH)
New Case MVA Viability Rate	High Consistency: Overall rate of MVA-positive results in new, untreated MB cases was similar.	75.4%	77.8%	75.0%
Transcript Profile Variability	High Variability: Proportion of MVA-positive cases expressing both transcripts (*hsp18*/*esxA*).	69.2% (Highest)	50.0%	44.5% (Lowest)
Molecular/Clinical Load	High Consistency: Strong, significant correlation between RLEP qPCR load and SSS BI (Bacillary Index) was found across all sites.	r=0.70	r=0.81	r=0.73

The validation of the MVA across the Philippines, Ethiopia, and Nepal confirms its status as a reliable and specific molecular tool for assessing *M. leprae* viability, offering a rapid, objective alternative to the MFP assay. However, the study reveals two key areas of significant geographic variability: the bacterial transcriptional profile and the MVA positivity rate among relapse cases. This regional divergence strongly suggests that the actual metabolic state of the viable bacilli and the corresponding host-pathogen dynamics may differ substantially across endemic settings.

4. Conclusion

The *Mycobacterium leprae* Molecular Viability Assay (MVA) represents a promising approach for assessing bacterial viability in leprosy patients. MVA is designed with high specificity unlike the current gold standard. By minimizing the risk of false positives and over-sensitivity to dead bacterial RNA, the MVA offers a specific and sensitive viability measurement. As a result, this assay can be used as a key supporting tool that can accelerate the reduction of leprosy prevalence and reliably predict the success of patient treatment. To fully establish its utility, however, it is advisable to conduct further validation studies across a wider and more diverse range of endemic populations before full-scale community implementation.

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