RESEARCH ARTICLE

Analysis of the Validity of Urine LAM ELISA for Tuberculosis Infection

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ABSTRACT

Objective: To explore the validity of urinary lipoarabinomannan (LAM) enzyme-linked immunosorbent assay (ELISA) assay technology for detecting MTB infection in the double infection of acquired immunodeficiency syndrome (AIDS) and human immunodeficiency virus-tuberculosis (HIV-TB) population with sputum-producing problems, and to explore the background value and medical reference value range of urinary LAM in the general population and people living with human immunodeficiency virus (HIV) or patients with acquired immunodeficiency syndrome (HIV/AIDS population).

Method: About 307 individuals from the general population group, HIV/AIDS population group, TB population group and HIV-TB population group provided by Seventh Hospital of Tangshan city were selected for early morning urine analysis. LAM ELISA competition method and double antibody sandwich method were used to detect the concentration of LAM in urine. Standard curves of LAM optical density OD value were drawn. The differences in LAM concentration in different groups of urine were calculated, and the diagnostic validity of LAM ELISA techniques was explored.

Result: (1) The corresponding curve formula of the ELISA competition method was $y = 1.696-0.087x+3.100/x^2$; The corresponding curve formula for the double antibody sandwich method was $y = -0.205+0.587x-0.097x^2+0.001x^3$. (2) In LAM ELISA competition method, the difference in LAM OD values between the TB population group and the general population group was statistically significant ($t = 3.393$, $p < 0.05$), and the difference in LAM OD values between the HIV-TB population group and the HIV/AIDS population group was statistically significant ($t = 2.294$, $p < 0.05$); The difference in LAM concentration between TB population group and general population group was statistically significant ($t = -4.642$, $p < 0.05$), and the difference in LAM concentration between HIV-TB population group and HIV/AIDS population group was statistically significant ($t = -4.737$, $p < 0.05$). In LAM ELISA double antibody sandwich method, there was a statistically significant difference in LAM OD values between TB population group and the general population group ($t = -2.566$, $p < 0.05$), and there was a statistically significant difference in LAM OD values between HIV-TB population group and HIV/AIDS population group ($t = -3.212$, $p < 0.05$); The difference in LAM concentration between TB population group and general population group was statistically significant ($t = -5.722$, $p < 0.05$), and the difference in LAM concentration between HIV-TB population group and HIV/AIDS group was statistically significant ($t = -8.118$, $p < 0.05$). (3) Receiver operating characteristic curve (ROC) curve analysis showed that in the LAM ELISA competition method, the SPE of TB infection in the HIV-TB population group diagnosed with urine LAM was higher than those in TB population group, with a statistically significant difference ($F = 31.227$, $p < 0.05$). Compared to the general population group, LAM ELISA competition method SEN in TB population group was lower than that in the ELISA double antibody sandwich method, and the difference was statistically significant ($F = 15.667$, $p < 0.05$).

Conclusion: The validity of urine LAM ELISA technology in the HIV-TB population group with TB infection was better than that in TB population group, and the validity of the LAM ELISA double antibody sandwich method was better than that in ELISA competitive method. The feasibility of urine LAM ELISA technology in HIV-TB was worthy of recognition, and the technology could be further improved and promoted.

Keywords: Tuberculosis, AIDS, Immunodeficiency virus, Mycobacterium tuberculosis complex, Lipoarabinomannan assay.

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Conflict of interest: None.

INTRODUCTION

On Nov. 7th, 2023, the World Health Organization (WHO) released the "Global TB Report 2023", which showed that TB would still be the world's second-largest infectious disease killer after COVID-19. In 2022, it was estimated that there would be 10.6 million new cases of TB worldwide, and 6.3% of those cases would be HIV infected.[1] Etiological laboratory assay methods for TB population include smear microscopy,

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MTB culture, and T lymphocytes γ-Interferon analysis, molecular amplification, etc.[2-8] In HIV/AIDS populations with immune disorders, samples with low levels of MTB bacteria may limit the validity of traditional etiological diagnosis. The report showed that from 2016 to 2020, the popularization rate of MTB molecular diagnostic technology in 11 prefecture-level city disease prevention and control center laboratories in Hebei Province reached over 90%, but the positive rates of MTB pathogen were below 36.28%. Especially among the HIV/AIDS population, although 92.37% have undergone TB infection screening, the MTB etiological positive rates were less than 15%.[9] Mannose-capped LAM is an important component and virulence factor on the cell wall of *Mycobacterium tuberculosis* (MTB). The WHO recommended it as one of the candidate biomarkers for detecting MTB antigens in 2014. [10] This study focused on exploring the validity of urinary LAM ELISA technology in detecting MTB infection in HIV and related populations in Tangshan and provided a theoretical reference for the feasibility study of this technology.

MATERIALS AND METHOD

Data Source

The samples for this study were collected from the Seventh Hospital of Tangshan City. Early morning urine was collected from 107 individuals in the general population group, 55 individuals in HIV/AIDS population group, 102 individuals in the population group, and 42 individuals in HIV-TB population group, totaling 306 individuals. TB patients were diagnosed according to "Tuberculosis Diagnosis (WS288-2017)", and HIV/AIDS was diagnosed according to "AIDS and AIDS virus Infection Diagnosis (WS293-2019)". The study was a retrospective study and had been approved by the theoretical committee.

Inclusion and Exclusion Criteria

Inclusion criteria: (1) People admitted to the hospital as suspected TB patients; (2) People received no treatment or less than 2 weeks of anti-TB treatment; (3) People Volunteered to participate and sign an informed consent form. Exclusion criteria: (1) People with severe kidney disease; (2) People for other reasons, such as long-term use of medication containing *M. tuberculosis* complex (MTBC) components.

Instruments and Reagents

Low temperature centrifuge JIDI-16R (Guangzhou Jidi Instrument Co., Ltd.), Enzyme marker Authos-340RT (Biochrom Company, UK), ELISA competitive assay kit (Andy Gene Co., Ltd.), ELISA double antibody sandwich assay kit (Andy Gene Co., Ltd.).

Sample Processing

Urine samples were collected in sterile tubes, centrifuged for 20 minutes (3000 r/min), and collect supernatant.

ELISA

- According to the instructions of the reagent kit, LAM ELISA competition method operation steps involved adding samples and standards, adding enzymes, incubating, preparing a solution, washing, developing color, terminating, etc. The corresponding concentrations of the five-point method standard curve were 12, 6, 3, 1.5, 0.75 ng/mL; the LAM ELISA double antibody sandwich operation steps method involved: adding samples and standards, incubation, solution preparation, washing, adding enzymes, warm bath, washing, developing color, terminating, etc. The corresponding concentrations of the five-point method standard curve were 9, 6, 3, 1.5, and 0.75 ng/mL. The standard sample concentration at each well position was measured 4 times and the average values were taken.
- The OD values of each standard and sample well were read at a wavelength of 450 nm.
- Standard curves were drown and the concentrations of LAM in different groups of samples were calculated.
- The urine LAM assay OD values and concentrations of the general population group, HIV/AIDS population group, TB population group, and HIV-TB population group were recorded.

Validity analysis

ROC curves were created and the sensitivity (SEN) and specificity (SPE) of LAM competition and sandwich methods were analyzed.

Statistical processing

SPSS 22.0 for routine statistical analysis, Curve Expert 1.4 for creating standard curves and calculating LAM concentration values at each sample well, and Medcalc15.0 software for plotting ROC curves and testing levels $\alpha = 0.05$.

Quality control

The collection of samples in this study strictly followed the "Collection and Processing of Human Urine Samples (GB/T 38735-2020)" and "Guidelines for Collection and Processing of Urine Samples (WS/T 348-2011)". Blank controls, standard controls, etc. were set up in the experiment, and two qualified operators completed the testing operation. The later data processing was completed through the blind method.

RESULTS

Standard Curve Construction

The corresponding OD values and LAM concentration results of LAM ELISA competition method and double antibody sandwich method standard curves were shown in (Table 1). "x"

Table 1: Corresponding OD values and LAM content results of LAM ELISA competition method and antibody sandwich method standard curves

Competition method		*Sandwich method*	
LAM (ng/mL)	*OD (x ± s)*	*LAM (ng/mL)*	*OD(x ± s)*
12.00	0.653 ± 0.005	9.00	1.712 ± 0.090
6.00	1.327 ± 0.021	6.00	1.158 ± 0.093
3.00	1.724 ± 0.035	3.00	0.858 ± 0.075
1.50	2.953 ± 0.062	1.50	0.468 ± 0.023
0.75	7.142 ± 0.087	0.75	0.189 ± 0.010

is LAM concentration, "y" is OD value. The corresponding curve formula of LAM ELISA competition method was $y = 1.696-0.087x+3.100/x^2$, and the results were shown in Figure 1; The corresponding curve formula for LAM ELISA double antibody sandwich method was $y = -0.205+0.587x-0.097x^2 + 0.001x^3$, and the results were shown in Figure 2. The relevant parameters and statistical results were shown in Table 2.

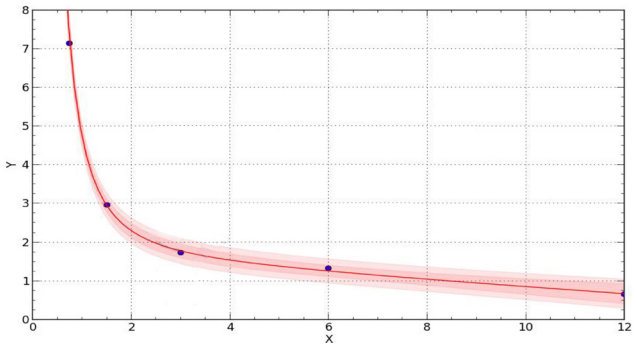

Figure 1: LAM ELISA competition method standard curve

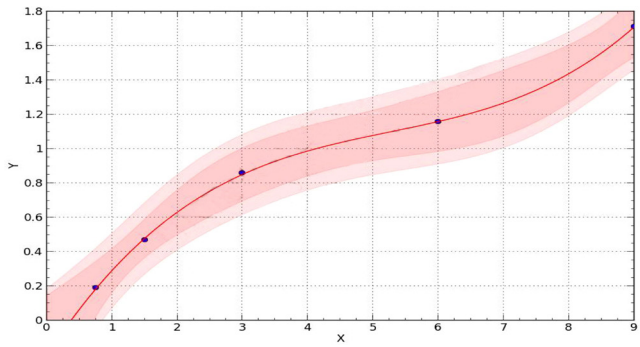

Figure 2: LAM ELISA double antibody sandwich method standard curve

Table 2: Relevant parameters of LAM ELISA competition method and double antibody sandwich method standard curves

	Competition method	*Sandwich method*
Name	Heat capacity	Polynomial regression
Type	Regression	Regression
Formula	$y = 1.696-0.087x+3.100/x^2$	$y = -0.205+0.587x-0.097x^2+0.001x^3$
standard errors ($S_{\grave{x}}$)	0.063	0.014
correlation coefficient(r)	1.000	1.000
Determination coefficient (r^2)	1.000	1.000

Analysis of LAM Concentration in Urine of Different Population Groups

LAM ELISA competition method and double antibody sandwich method were used to determine the OD value and LAM concentration in the general population group, HIV/AIDS population group, TB population group, and HIV-TB population group, as shown in (Table 3).

Analysis of LAM OD Value and Concentration in ELISA Competition Method

There was a statistically significant difference in OD values between TB group and the general group ($t = 3.393$, $p < 0.05$), and the differences in OD values between the HIV-TB group and the HIV/AIDS group was statistically significant ($t = 2.294$, $p < 0.05$). The results were shown in (Table 4); The difference in LAM concentration between TB group and the general group was statistically significant ($t = -4.642$, $p < 0.05$), and the difference in LAM concentration between the HIV-TB group and HIV/AIDS group was statistically significant ($t = -4.737$, $p < 0.05$). The results are shown in Table 5.

Analysis of LAM OD Value and Concentration in ELISA Double Antibody Sandwich Method

There was a statistically significant difference in OD values between TB group and the general group ($t = -2.566$, $p < 0.05$), and the difference in OD values between the HIV-TB group and HIV/AIDS group was statistically significant ($t = -3.212$, $p < 0.05$). The results were shown in (Table 6); The difference in LAM concentration between TB group and the general group was statistically significant ($t = -5.722$, $p < 0.05$), while the difference in LAM concentration between the HIV-TB group and HIV/AIDS group was statistically significant ($t = -8.118$, $p < 0.05$). The results are shown in Table 7.

Validity Analysis

ROC curve analysis

In LAM ELISA competition method, the ROC curve corresponding to the OD value of TB population group relative to general population group was shown in Figure 3, and the ROC curve corresponding to the OD value of the HIV-TB population group relative to HIV/AIDS population group was shown in Figure 4; In LAM ELISA double antibody sandwich

Table 3: Urine LAM OD value and concentration using LAM ELISA competition method and double antibody sandwich method for different populations

	Competition method					*Sandwich method*				
	OD		*LAM concentration*			*OD*		*LAM concentration*		
	$\grave{x}$	*Q*	$\grave{x}$	*S*	$S_{\grave{x}}$	$\grave{x}$	*Q*	$\grave{x}$	*S*	$S_{\grave{x}}$
General population (n = 107)	2.675	1.603	3.454	1.748	0.169	1.059	0.992	3.993	2.686	0.260
HIV/AIDS population (n = 55)	2.071	1.403	3.699	1.648	0.222	1.098	1.088	3.867	1.769	0.238
TB population (n = 102)	1.764	1.438	4.832	2.464	0.244	1.470	1.154	6.306	3.151	0.312
HIV-TB population (n = 42)	1.477	1.440	6.320	3.282	0.506	2.587	1.608	8.870	3.682	0.568

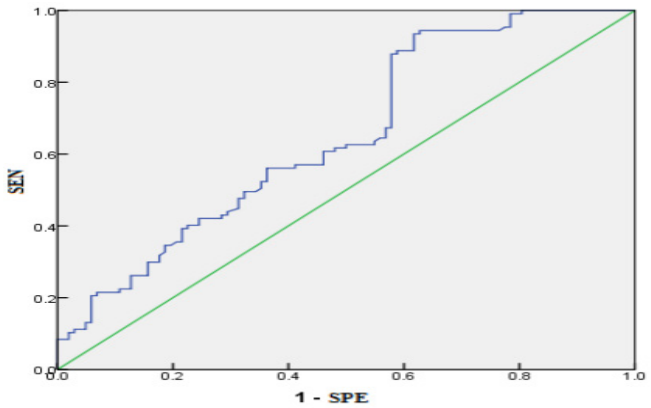

Figure 3: The ROC curve corresponding to LAM ELISA competition method OD value in TB population group compared to the general population group

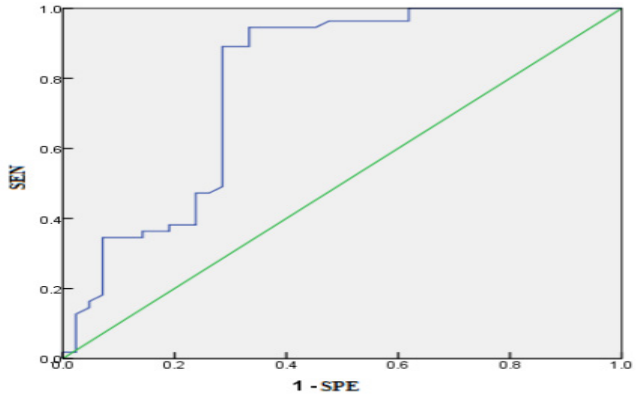

Figure 4: The ROC curve corresponding to LAM ELISA competition method OD value in HIV-TB population group compared to the HIV/AIDS population group

Table 4: Differences in LAM OD values in urine by LAM ELISA competitive method among different populations

	HIV/AIDS population		*TB population*		*HIV-TB population*	
	t	*P*	*t*	*P*	*t*	*P*
General population	1.954	0.052	3.393	0.001	3.906	0.000
HIV/AIDS population			1.445	0.152	2.294	0.024
TB population					1.339	0.185

Table 5: Differences in LAM content in urine by LAM ELISA competitive method among different populations

	HIV/AIDS population		*TB population*		*HIV-TB population*	
	t	*P*	*t*	*P*	*t*	*P*
General population	-0.877	0.382	-4.642	0.000	-5.366	0.000
HIV/AIDS population			-3.432	0.001	-4.737	0.010
TB population					-2.646	0.010

Table 6: Differences in LAM OD values in urine by LAM ELISA sandwich method among different population groups

	HIV/AIDS population		*TB population*		*HIV-TB population*	
	t	*P*	*t*	*P*	*t*	*P*
General population	-0.179	0.858	-2.566	0.011	-3.521	0.001
HIV/AIDS population			-1.626	0.107	-3.212	0.002
TB population					-2.549	0.014

Table 7: Differences in LAM concentration by LAM ELISA sandwich method among different population groups

	HIV/AIDS population		*TB population*		*HIV-TB population*	
	t	*P*	*t*	*P*	*t*	*P*
General population	0.354	0.724	-5.722	0.000	-7.808	0.000
HIV/AIDS population			-6.209	0.000	-8.118	0.000
TB population					-3.956	0.000

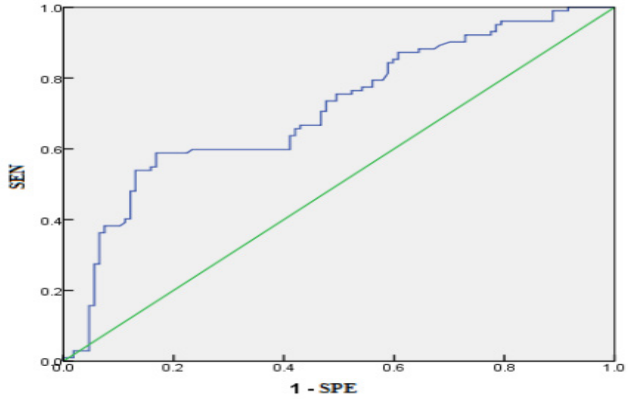

Figure 5: The ROC curve corresponding to LAM ELISA sandwich method OD value in TB population group compared to the general population group

method assay, the ROC curve corresponding to the OD value of TB population group relative to general population group was shown in Figure 5, and the ROC curve corresponding to the OD value of HIV-TB population group relative to HIV/AIDS population group was shown in Figure 6. The area under the ROC curve (ROC AUC) and cut off value (CO value) of different populations and assay methods were shown in Table 8.

LAM ELISA competition method SEN and SPE Analysis

The SEN of TB infection by LAM ELISA in HIV/AIDS population was higher than that in the general population, with no statistically significant difference ($F = 0.023$, $p > 0.05$); The SEN of TB infection by urine LAM ELISA in HIV/AIDS population was higher than that in the general population, with a statistically significant difference ($F = 31.227$, $p < 0.05$).

LAM ELISA double antibody sandwich method SEN and SPE analysis

The SEN of TB infection by LAM ELISA in HIV/AIDS populations was higher than that in the general population, with no statistically significant difference ($F = 1.283$, $p > 0.05$). The SPE of TB infection by LAM ELISA in HIV/AIDS patients was higher than that in the general population, with no statistically significant difference ($F = 0.080$, $p > 0.05$).

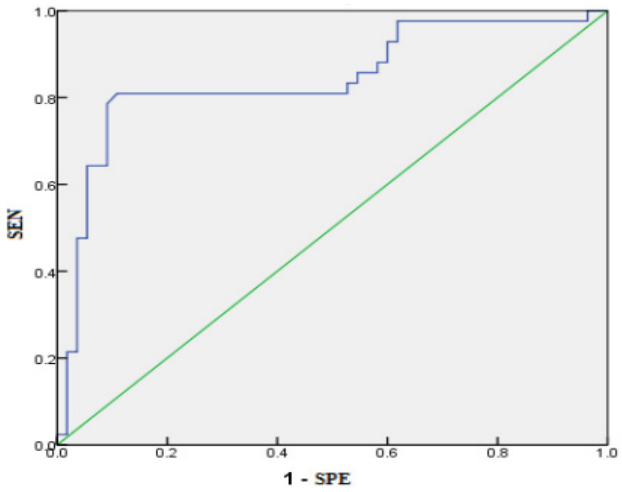

Figure 6: The ROC curve corresponding to LAM ELISA sandwich method OD value in HIV-TB population compared to the HIV/AIDS population

Table 8: LAM ELISA ROC AUC and CO values for different populations and different assay methods

		Competition method	*Sandwich method*
TB population to general population	ROC AUC (CI)	0.650 (0.576~0.725)	0.712 (0.641~0.782)
	CO value	1.345	1.106
	SEN (%)	18.627 (19/102)	58.824 (60/102)
	SPE (%)	98.131 (105/107)	83.178 (89/107)
HIV-TB population to HIV/AIDS population	ROC AUC	0.787 (0.687~0.887)	0.842 (0.756~0.928)
	CO value	1.355	1.110
	SEN	66.667 (28/42)	80.952 (34/42)
	SPE	96.364 (53/55)	89.091 (49/55)

LAM ELISA of SEN and SPE analysis in different populations

TB population group compared to the general population group, LAM ELISA competition method SEN was lower than that in the double antibody sandwich method. The difference was statistically significant ($F = 15.667$, $p < 0.05$); LAM ELISA competitive method SPE was higher than that in the double antibody sandwich method, with no statistically significant difference ($F = 0.693$, $p > 0.05$). HIV-TB population group compared to HIV/AIDS population group. LAM ELISA competition method SEN was lower than that in the double antibody sandwich method, and the difference was not statistically significant ($F = 0.335$, $p > 0.05$); The SPE of LAM ELISA competition method was higher than that in LAM ELISA double antibody sandwich method, and the difference was not statistically significant ($F = 0.081$, $p > 0.05$).

DISCUSSION

The WHO recommended in 2016 the application of lateral flow LAM assay for CD4+T lymphocytes<100/μL HIV positive TB patients.[11] According to relevant reports, LAM assay technology was mostly used for serum samples from immuno-compromised populations for TB infection at the early stage.[12,13] This study focused on the assay of LAM in morning urine from different populations. The sampling method was easy to grasp, the assay process was easy to operate, and the assay results could be quantitatively analyzed.

In this study, both urinary LAM ELISA techniques were able to construct a relatively complete and smooth standard curve through the measurement of absorbance after standard dilution. The stability and repeatability of the experiment were confirmed by the mean and standard deviation results of four repeated measurements. The optimal curves and related formulas $y = 1.696\text{-}0.087x + 3.100/x^2$ and $y = -0.205 + 0.587x - 0.097x^2 + 0.001x^3$ drawn by Curve Expert software also fit the classical curve equations of ELISA competition method and double antibody sandwich method well.[14,15] The determination coefficients r^2 of both curves were greater than 0.99, providing quality assurance for the overall linear range of LAM ELISA in different populations in the later stage.

The study found significant differences in LAM OD values and concentrations between the TB population group and the general population group in the LAM ELISA competitive method and the double antibody sandwich method. There are also significant differences in LAM OD values and concentrations between the HIV-TB population group and the HIV/AIDS population group. The background value of LAM content in the competitive LAM ELISA method for the general population was 3.454 ± 1.96*1.748, while the corresponding background value for the double antibody sandwich method was 3.993 ± 1.96*2.686, and the results were basically consistent.

At the same time, the research results showed that the medical reference value range of the ELISA competitive method for LAM content in the TB population was 4.832 ± 1.96*2.646, while the medical reference value range of the double antibody sandwich was 6.306 ± 1.96*3.151, indicating significant differences in the results; The medical reference value range for LAM content in HIV-TB population using ELISA competitive method was 6.320 ± 1.96*3.282, while the corresponding medical reference value range for ELISA double antibody sandwich method was 8.870 ± 1.96*3.682, indicating significant differences in the results. The above results provided numerical interval references for the clinical application of the two LAM ELISA experimental techniques and exploration space for further validity analysis of different ELISA methods in the later stage.

By plotting the ROC curve, we found that the overall assay validity of the LAM ELISA competitive method was lower than that of the double antibody sandwich method. Analyzing the reasons, the ELISA competitive method was suitable for detecting antigen molecules with small molecular weights and relatively single surface determinant clusters, while the ELISA double antibody sandwich method was suitable for antigen molecules with larger molecular weights.[16] LAM belongs to the class of lipopolysaccharides, with a relative molecular weight of about 17.30 kDa. It belonged to the macromolecular type of end-connected mannose (Man),

arabinose, and methylthio-D-xylose (MTX). The key to its epitope length and structural composition was the epitope structure's configuration and the linkage with Man and MTX branches.[17-19] The dynamic changes in these structures would have an impact on the recognition of LAM epitopes, thereby affecting the results of ELISA experiments, especially in the assay of LAM ELISA competitive method SEN. However, LAM molecules themselves had strong thermal stability and could be stored for a long time,[20] which were not easily degraded during the preservation process of clinical samples, thus ensuring the LAM ELISA competition method and double antibody sandwich method SPE.

Through the observation of ROC curves, we also found that the two LAM ELISA techniques have better TB assay validity in HIV/AIDS populations than in non-HIV/AIDS populations. Analyzing the reasons, it might be due to varying degrees of immune damage in the HIV/AIDS population, and high MTB load and systemic transmission might lead to high levels of LAM expression in urine. Related reports had shown that urine LAM testing was particularly suitable for critically ill patients with concurrent HIV infection and low CD4+T lymphocyte count. The SEN of urine LAM testing might be negatively correlated with CD4+T lymphocyte count. For patients with CD4+T lymphocyte count less than 50 cell/mL, the SEN of urine LAM assay could reach 56 to 85%, and the SPE could reach 88%.[21,22] It had also been found that HIV-1 TAT protein can change the cytoskeleton and renin distribution of human kidney podocytes and improve the glomerular permeability, which might also be the reason for the high level of LAM in the urine of HIV-positive TB patients.[23,24]

Related reports show that the SEN of serum LAM assay in early clinical trials was lower than 20.00%.[25] The serum LAM assay SEN for TB infection in HIV/AIDS populations in South Africa, Vietnam, and Ghana was 34.90%. Among TB patients with $CD4^{+}T < 100$ cell/mL, the SEN was 56.00%.[26] The Fuji LAM urine assay SEN in Japan was 67.40%.[27] The photon biosensor LAM technology had exceeded 90.00% in SEN and SPE in TB assay.[28, 29] However, the continuous increase in testing costs had limited the application and promotion of LAM as a clinical diagnostic biomarkers.[30]

This study aims to explore efficient and low-cost LAM assay methods. In the later stage of research, the research team will further improve the extraction method and assay signal interpretation of urinary LAM samples, thereby increasing the laboratory assay rate of MTB infection in HIV/AIDS populations, especially those with sputum production disorders. Then, the scientific reference for the implementation of the "early assay, early reporting, early isolation, early treatment, and early screening" strategy for the TB population will be provided.

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