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Short communication

A multiplexed nucleic acid microsystem for point-of-care detection of HIV co-infection with MTB and PCP

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ABSTRACT

Many individuals infected with the human immunodeficiency virus (HIV), especially children in African countries, die of co-infections with *Mycobacterium tuberculosis* (MTB) (coinfection rate: 50%) or *Pneumocystis carinii pneumonia* (PCP) (coinfection rate: 81%). The present proposal describes a rapid, portable, low-cost, multiplexed point-of-care diagnostic technique for simultaneously detecting HIV, MTB, and PCP. This technique incorporates a creative micro-device (hardware) and a loop-mediated isothermal amplification strategy (software).

1. Introduction

Since the discovery of AIDS in the 1980s, the illness has spread extremely rapidly and has come to seriously threaten human survival and development [1]. This disease is caused by the human immunodeficiency virus (HIV) [2]. However, HIV infectees often do not die of the disease itself but rather from co-infection with other pathogens, such as *Mycobacterium tuberculosis* (MTB) or *Pneumocystis carinii pneumonia* (PCP). MTB is the most common opportunistic infection pathogen and killer of AIDS patients, and MTB and HIV co-infections have an extremely high mortality and fatality rate [3–5]. Reports indicate that one in three HIV infectees in the world is infected with MTB, especially in certain remote developing areas, such as certain poor Africa countries. Nearly half of individuals infected with HIV are infected with pulmonary tuberculosis, especially in China. Once HIV infectees become co-infected with pulmonary tuberculosis pneumonia, the possibility of development into active pulmonary tuberculosis is 30–50 times higher than in individuals not infected with HIV. *Pneumocystis carinii pneumonia* (PCP) occurs mainly in immunocompromised children. Heather Zar and colleagues from the University of Cape Town have reported that, in South Africa, *Pneumocystis carinii pneumonia* is common among HIV-infected infants, causing nearly 50% mortality [6,7]. To the best of our knowledge, the standard detection assay of the HIV, MTB or PCP was mainly based on the immunoassays, (such as enzyme-linked immune sorbent assay or gold-immunoassays) and the common fluorescent PCRs. Although the wide application of the gold-immunoassays, because of its easy-to-use, rapid-to-detection and low-cost, the detection limit should be future improved to optimize the diagnostic effect. In addition, the immunoassay-based diagnostics was to not good enough to the condition of the "window phase" for the detecting pathogens. PCR-based nucleic acids detection for the HIV etc. was not a situble method to the point-of-care applications in the remote country because of its high-cost and complexity.

In the present report, microfluidics and loop-mediated isothermal amplification was used to develop a rapid, portable, low-cost, multiplexed point-of-care diagnostic technique for simultaneously detecting HIV, MTB, and PCP for the diagnosis and prevention of these diseases [8–10].

2. Methods

2.1. Materials

Conserved nucleic acid fragments of HIV, PCP, and MTB were screened and cloned into the PUC57 plasmid. Plasmids containing the target nucleic acid fragment were used as standards. The reference strains of these three pathogens were prepared in the lab. Sample of HIV and PCP were provided with a commercially available diagnostic kit. *M. tuberculosis* (MTB) from a cell culture

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was provided by the Shanghai Public Health Clinical Center (Shanghai, China). Total genomic DNAs/RNAs were extracted using QIAamp® DNA Blood Mini Kit (Qiagen GmbH, Germany) or Trizol Kit (Invitrogen, U.S.).

2.2. LAMP establishment

LAMP was carried out in a system containing 1 × ThermoPol Buffer (20 mM Tris–HCl, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1%Triton X-100, pH 8.8 at 25 °C), 8.0 mM $MgSO_4$, 1.0 mM dNTPs, 0.2 μM each of the outer primer (F3 and B3), 1.6 μM each of inner primer (FIP and BIP), and 0.8 μM each of loop primer (LF and LB), 0.2 U/μL of AMV transcriptase, and 0.32 U/μL of *Bst* polymerase with a small amount of sample. Amplification was performed at 63 °C in a water bath (or thermobaker) for 1 h followed by agarose gel electrophoresis. The final result was determined by the naked eye and based on the appearance of white precipitate of magnesium pyrophosphate and the green fluorescence induced by the intercalating dye SYBR green I [11,12].

2.3. Multiplex LAMP chip fabrication and manipulation

A master of chip in the pattern of a five-pointed star was fabricated by mechanical microfabrication. A PDMS replica was produced by molding, as described previously [12,13]. The PDMS replica was sealed with a microscope slide before plasma treatment. The 0.6 μL different solutions, each of which contains a specific LAMP probe set (quantities of probe: F3/B3, 1 pmol; FIP/BIP, 8 pmol; LF/LB, 4 pmol) were injected into corresponding microchannels via the end outlet and then evaporated completely, leaving the probes in the microchamber.

The 2 μL sample and 23 μL LAMP reaction buffer were sequentially introduced into the system via the center hole and the five microchambers were completely filled by capillary force. The inlet/outlet was then tightly sealed with the uncured PDMS to form an integral device. The whole chip was incubated at 63 °C for 1 h using a water bath and the final results were determined using the naked eye or analyzed by agarose gel electrophoresis [14].

3. Results and discussion

A relatively conserved target sequence of the pathogens of HIV, MTB, and PCP was found out by homologous analysis of gene sequence using Vector NTI software. The results are shown in Table 1. The homology of these conserved nucleic acid sequences was above 90%. The pathogens could be identified through amplification of these conserved nucleic acid sequences by specific LMAP primers. The LAMP primer used here was designed using Primer Designer V3 [11].

The multiple LAMP microfluidic chip has amplification pools in the shape of a five-pointed star. Each pool is connected to the diffusion-limited capillary channel via a connecting pipe. There is a hole in the middle of the chip through which the sample is distributed to the five amplification pools by capillary force and then amplified by LAMP (Fig. 1a). Due to the small dimensions of the apparatus, the diffusion-limited capillary channel has a very small diffusion constant, which effectively prevented the diffusion of LAMP nucleic acid probe and reaction products in different amplification pools. A connecting pipe is used to connect the amplification pool and the diffusion-limited capillary channel so that the sample or reaction liquid can spread evenly through the amplification pools. Multiple targets can be detected simultaneously in one sample by spatial differentiation of amplified signal with series of spatial ordered amplification pools coated with specific nucleic acid probes [18–20].

Table 1
Nucleotide sequence of gene markers of three influenza A subtypes.

Viruses	LAMP primers
HIV[15]	F3: ATTATCAGAAGGAGCCACC
	B3: CATCCTATTTGTTCCTGAAGG
P24 gene	FIP: CAGCTTCCTCATTGATGGTTTCTTT TTAACACCATGCTAAACACAGT
	BIP: TGTTGCACCAGGCCAGATAATTTT GTACTGGTAGTTCCTGCTATG
	LF: TTTAACATTTGCATGGCTGCTTGAT
	LB: GAGATCCAAGGGGAAGTGA
MTB[16]	F3: GCGATATCTGGTGGTCTGCA
	B3: GGCACCAAAGCTTTTGTCGC
Gyr B gene	FIP: TCTGGTGAGCATGGGCAGCG CCGGTGGTTAACGCGCTAT
	BIP: TATGAGAAGTCGGAACCCCTGG GTGGCAACTGGGGCAGAAGAA
	LF: AACTAGAGCTGAAGCTCGG
	LB: CCTCAAGCAAGGGGCGCC
PVP[17]	F3: AGCGTATATTAAAGTTGCTGC
	B3: GCTTTGAACACTCTAATTTTCTCA
18S rRNA gene	FIP: FIPCCAGTGCACACACTTCGGAGTTA AAAAGCTCGTAGTTGAACCTA
	BIP: GCGATCCTTCCTTCTGGATTACAA GTAAAAGGCCCTGGTTA
	FL: GACCGGGCCGTCAAC
	BL: GTATGCCCTTCATTGGGTGTAT

In order to construct functional multiplex LAMP chips capable of detecting HIV, MTB, and PCP, five amplification pools were coated with specific LAMP probes for these three kinds of pathogens: amplification pools 1, 2, and 3 were coated with HIV-LAMP probe, MTB-LAMP probe, and PCP-LAMP probe, respectively. Amplification pool 4 served as a positive control and was coated with β-actin LAMP probes and amplification pool 5 served as a negative control and was left uncoated [20]. In the experiment, 2 μL nucleic acid sample and 23 μL LAMP reaction buffer were sequentially introduced into the system. It should be noted that if real clinical samples was used, for example the blood of infectee, the whole process of sample pretreatment and nucleic acid extraction/purification should be carried out in the level three biological laboratory to avoid potential infection to the human being. While in our current study, the extraction of nucleic acids could be extracted by QIAamp® DNA Blood Mini Kit (Qiagen GmbH, Germany) or Trizol Kit in common laboratory. Because we have used the standard and artificial samples. Once the amplification pools were completely filled with LAMP reaction buffer, the inlet/outlet was tightly sealed with uncured PDMS. The whole chip was then incubated at 63 °C for 1 h in a water bath or using another heat source. Solar energy may be a sufficient heat source, which would make this test usable in areas without electricity. The final results were assessed using the naked eye or analyzed using agarose gel electrophoresis. Naked eye detection was based on the appearance of white magnesium pyrophosphate precipitate generated during the LAMP reaction and the green fluorescence induced by the intercalating dye SYBR green I. The chip spectrum of HIV co-infection with MTB or PCP infection and infection with HIV alone are shown in Fig. 1a–c. The corresponding amplified LAMP product was determined by agarose gel electrophoresis (Fig. 1e). The electrophoretical pattern of LAMP product was different from the typical PCR pattern, which appears as ladder-like bands. Results indicated that the LAMP microchip can be used for multiple detection of pathogens.

Sensitivity, specificity and accuracy are key parameters to evaluate a novel detection system. In this study, the sensitivity of the LAMP chip was evaluated using serial dilutions of each of the target nucleic acid samples (10^5, 10^4, 10^3, 10^2, 50, and 0 copies/μL). Results are shown

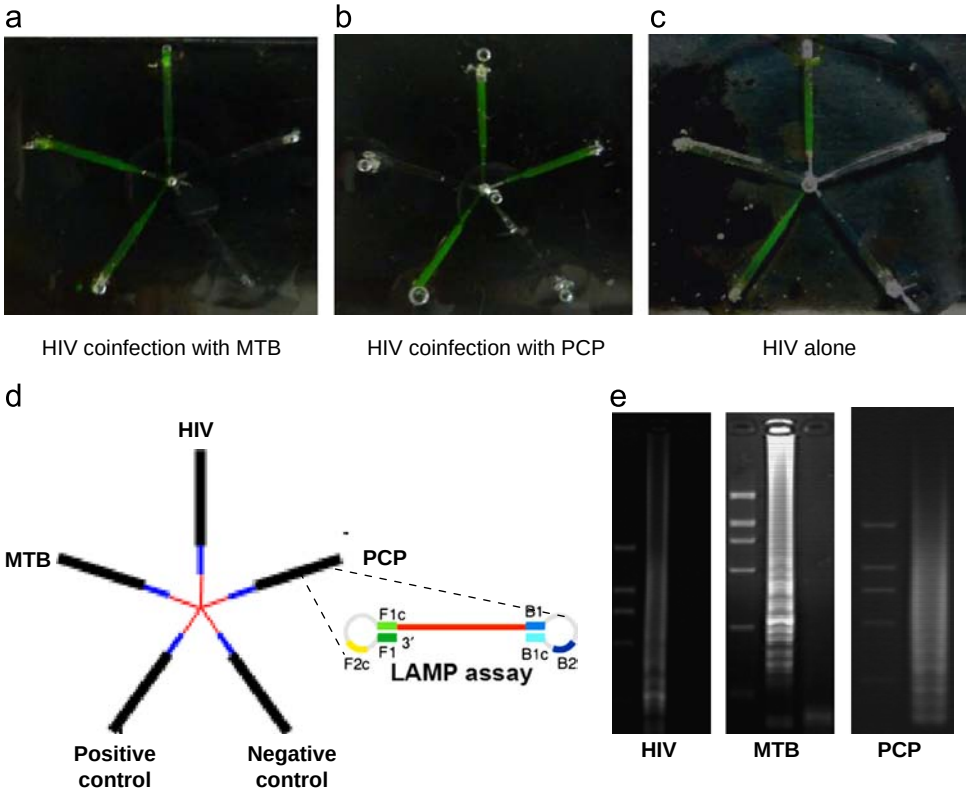

Fig. 1. Five-channel μLAMP for point-of-care analysis of HIV, MTP, and PCP. (a) Spectrum of HIV, MTB coinfection. (b) Spectrum of HIV PCP coinfection. (c) Spectrum of HIV infection alone. (d) Five-channel LAMP microfluidic chip. (e) Gel electrophoresis spectrum of LAMP amplification of HIV, MTB, and PCP.

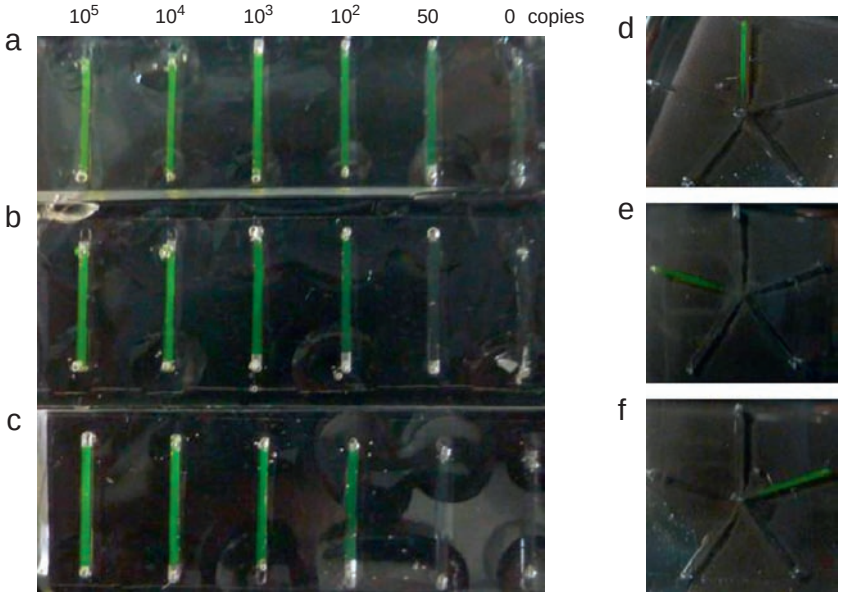

Fig. 2. Performance of LAMP chip in the detection of three pathogens. (a–c) Sensitivity of detection of HIV, tuberculosis bacilli, and Karsh of *Pneumocystis carinii* μLAMP chip. (d–f) Specificity of detection of HIV, tuberculosis bacilli, and Karsh of *Pneumocystis carinii* μLAMP chip.

in Fig. 2(a–c). These results indicate the detection limit of this multiplex LAMP chip to HIV, MTB, and PCP virus to be 50 copies, 100 copies, and 100 copies, respectively, which was about 100 orders of magnitude better than that of typical PCR method and comparable to the sensitivity of fluorescent PCR. Samples of HIV, MTB, and PCP were used to evaluate the specificity of the LAMP chip. Results are shown in Fig. 2(d–f). Samples of each specific pathogen showed specific amplification patterns and no other non-specific amplifications appeared. Genomic nucleic acids for swine fever virus, porcine reproductive and respiratory syndrome virus, pseudorabies virus, porcine transmissible gastroenteritis virus, porcine parvovirus and porcine circovirus virus, and the influenza a H1N1 influenza virus were used as templates to further confirm the non-cross reactivity of the LAMP chip (These samples were stored in our lab).

The accuracy (false positive and false negative rate) of the LAMP-based assay mostly depended on the specific primers used in the reaction system, while these primers we applied here have been comprensively evaluated in previous work. From the date of

Table 2
Comparison of multiplex LAMP chip to related techniques.

Related technology	Reusability	Readout	Instrument	Operation
Multiple LAMP chip	flexible and adjustable	naked eye	none	simple
Multiple PCR [21]	three times or fewer	fluorescence	fluorescence quantitative PCR	relatively simple
Gene chip [22]	more than 100 times	fluorescence	chip scanning instrument	complex
Flow fluorescence [23]	more than 100 times	fluorescence	Flow fluorescence	complex

these studies, the false positive and false negative rate could be accepted in real clinical practice, which was comparable to the standard method of PCR-based assay.

In all experiments, tests for pathogens were performed three times to confirm the reproducibility of the system and provided much more stable test results. Generally speaking, nucleic acid-based assay would be much more suitable for the pathogens detection than common protein-based assays. This is because the existence of the so called "window phase" of the pathogens. To our knowledge, the window phase of the pathogens is ~3 month, such as HIV. While the nucleic acid-based assay could dramatically shorten the window phase of the pathogen infection to the ~1 week. Which would be of very important to the infectee. From the view of test time, the whole test from the sample collecting to the answer readout of our device could be finished within 90 min, which would be much faster than current assays, including PCR-based assay, immunoassay and so forth. Apart from the rapid of our assay, other benefits when compared with typical tests were listed in Table 2. All above, all of the traits of rapid, low-cost, equipement-independent and the high accuracy of this method make it very suitable in the application of the pathogen detection in poor countries and areas.

4. Conclusion

In the present work, a microfluidic isothermal amplification chip incorporating loop-mediated isothermal nucleic acid amplification and the microfluidic technique was developed. This method can simultaneously detect HIV, MTB, and PCP. The micro-chip system developed in this report was highly sensitive and specific, fully capable of meeting the demand for a practical method of diagnosing these infections in clinical situations. It has considerable significance for developing countries, where a rapid, practical, multiple-target-point, low-cost molecular diagnostic technique for the diagnosis and resistance of disease of HIV, MTB, and PCP would facilitate timely diagnosis and treatment. In the present study, a variety of LAMP microfluidic techniques were used for the rapid detection of these pathogens.

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