ORIGINAL ARTICLE

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Comparison of Reverse Transcriptase Loop-Mediated Isothermal Amplification and Reverse Transcriptase Polymerase Chain Reaction for Detection of Prostate Specific Antigen

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ABSTRACT

Background: Research shows that prostate cancer ranks second among the top five most common cancers in men. It has been confirmed that when circulating Prostate Specific Antigen (PSA) transcripts are successfully detected, prostate cancer cells can be diagnosed at an early stage. A reverse transcriptase loop-mediated isothermal amplification (RT-LAMP) assay was developed and compared to reverse transcriptase polymerase chain reaction (RT-PCR) assay for detection of PSA.

Methods: 47 patients, including 30 patients with prostate cancer, 15 with Benign Prostate Hyperplasia (BPH) and 2 healthy subjects as negative controls were included in this study. The prostate cancer cell lines (PC3 and LNCaP) of two patients were included in the study as positive controls. Next, RNA was extracted from fresh samples and a first strand cDNA synthesis kit was applied for the synthesis of cDNA. The human prostate specific antigen gene was used to design specific primers.

Results: The results indicated that the control subjects and participants suffering from BPH were not positive. 13 out of 15 (86.6%) patients suffering from localized cancer were PSA positive. PSA positive results were observed among all 15 metastatic patients and positive controls (100%). RT-LAMP is an advantageous method because it is highly sensitive (1000-fold), quite cheap, user-friendly, and safe; in addition, it can be quickly performed by visual detection using GineFinder™ dye in a water bath.

Conclusion: RT-LAMP technique can be simply and reliably applied with the aid of basic instruments, and its results can be visually inspected in laboratory studies.

Keywords: Benign prostate hyperplasia, Prostate cancer, Prostate specific antigen, RT-LAMP assay, RT-PCR assay

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INTRODUCTION:

A large number of men die in the United States due to prostate cancer each year[1]. Following lung cancer, prostate cancer has become the most rampant cancer in men; every year, up to 300,000 new cases of prostate cancer are reported worldwide[2,3]. It is believed that measuring serum prostate specific antigen (PSA) levels help diagnose prostate cancer at an earlier stage[4]. There is no doubt that PSA successfully detects Prostate Cancer (CaP) in me, but elevated levels of PSA are also reported in men diagnosed with Benign Prostate Hyperplasia (BPH) and other prostate disorders[5]. Thus, it is necessary to use specific molecular markers to differentiate between CaP and other nonmalignant diseases. It has been reported that if circulating PSA transcripts are successfully detected, prostate cancer cells could quickly be identified[6,7]. Prostate epithelial cells produce PSA; it is a 34 kDa serine protease, which is classified as the third member of the human glandular kalikrein family hk1, hk2 and hk3[8]. High levels of hk2 expression has been observed in the prostate[9]. At concentrations of 0.5-3 mg/ml (i.e., about 1 million times the concentration seen in plasma), PSA enters the seminal fluid[10]. Following ejaculation, PSA aids in dissolving gel-forming proteis by digesting seminogelin 1, seminogelin 2 and fibronectin, leading to a consequent release of sperm; this process is believed to be necessary for sperm function[11]. PSA release is decreased by natural barriers (including the epithelial and basal cell layer as well as the basement membrane) in the prostate of healthy individuals prostate. Disruption of the basement membrane and the basal cell layer occurs in prostate carcinoma[12]. Therefore prostate epithelial cells become disorganized, possibly permeating the nearby stroma. Consequently, higher levels of PSA are secreted into the blood of prostate cancer patients[13]. Polymerase chain reaction (PCR)-based assays are the best alternative to PSA definitive diagnosis; however, its usefulness may be limited by its need for trained staff working with reagents and equipment in a professional operating space[14,15]. Recently, isothermal amplification methods providing simple and cost-effective molecular tests in low resource settings have been developed, with very high demand[16]. Loop-mediated isothermal amplification (LAMP) is one of these methods, which has been widely adopted today. In a single-step reaction, it can provide amplification of 109 copies out of a few copies of the target molecule in less than an hour, even in the presence of large amounts of non-target DNA[17]. LAMP can be thwarted by the Bst DNA polymerases (Thermostabile enzyme isolated from Bacillus smithii bacteria) commonly present in clinical samples and insects as they are more tolerant to the inhibitors[18]. Nevertheless, amplification based on LAMP assay can be simply done via visual detection of turbidity created by magnesium pyrophosphate precipitation, intercalating dye fluorescence, or color variations of metal-sensitive indicators by setting a simple electric device like a water bath or heat block at a constant temperature[19]. In the present study, we developed a RT-LAMP assay based on the human prostate specific antigen gene of PSA via a visualized system of detection. We also evaluated the efficiency, speed and sensitivity of RT-LAMP for PSA and compared its performance to RT-PCR.

METHODS:

Patients and Samples

By studying clinical and pathological records and focusing on adenocarcinoma of the prostate or BPH at the urology clinics of Shariati, Mehrad and Sina Hospitals, Tehran University of Medical Sciences, Iran, all the patients participating in the study (excluding control subjects) were selected. The study population consisted of 47 patients (15 with localized cancer, 15 with metastatic and 15 with BPH) as well as 2 healthy subjects (1 female and 1 male) as negative controls; their mean

age was 65.2 years (ranging from 50-80). The Iranian Pasteur Institute provided the study with two human prostate cancer cell lines, PC3 and LNCaP, which were used as positive controls. Biopsy was performed on all the patients (excluding the control subjects) due to their abnormal digital rectal exam (DRE) and high serum PSA level. Blood samples were taken (six ml peripheral blood) from all patients either immediately before the radical prostatectomy in the operation room or before biopsy; sodium citrate was applied to treat the blood samples. Following the International Prostate Symptom Score (IPSS) standard, a questionnaire was administered to the patients. Centrifuge was performed on the freshly obtained samples for 15 min at 10000 rpm. The next step was transferring the buffy coat to a new tube; an equal volume of phosphate buffer saline (PBS) was used once in order to wash the buffy coat.

RNA extraction and cDNA synthesis

Some minor modifications were made after the process of total RNA extraction of the samples20. A first-strand cDNA synthesis kit (Roche Inc., GMBH, Germany) was used for cDNA synthesis. Following the kit instructions, a small amount of reaction mix was achieved; it contained 2 µl dithiothreithol (DTT), 4 µl 5x buffer, 1 µl dNTP (10 mM), 1 µl oligo deoxy thymidine (dt), 1 µl random hexamer, 0.15 µl RNase inhibitor; 1.5 µl moloneymurine leukemia virus reverse transcriptase enzyme (MMLV) and approximately one microgram total RNA. Following the addition of H_2O, the volume of the mixture reached 25 µl. Synthesis of cDNA was done by the application of a programmable thermocycler for 55 min at 45 ºC; which was then incubated for 3 min at 95ºC. Finally, equilibrium was reached at 20 ºC for 1 min.

Primer design

The Oligo7 and Primer Explorer V.4 software were utilized to design RT-PCR and RT-LAMP specific primers based on the human prostate specific antigen gene (GenBank: M27274.1) (**Table 1**). They were subsequently examined based on sequence alignments using ClustalX 2.11 (Des Higgins). The positions of the designed primers on the sequence are displayed in **Figure 1A**.

RT-PCR assay

RT-PCR amplification was carried out in a thermocycler (iCycler, BIO RAD, CA, USA) using cDNA. The cDNA was incubated at 95 °C for 3 min and then chilled on ice for 3 min. Amplification was done in a volume of 25 µl containing 10 × PCR buffer (10 mM Tris-HCl, pH 8.3 and 50 mM KCl), 1.5 mM $MgCl_2$ (CinnaGen Co., Iran), 0.5 µM each of F and B primers, 0.2 mM of dNTPs (CinnaGen Co., Iran), 2 U of Taq DNA polymerase (CinnaGen Co., Iran) and 2 µl template cDNA. Amplification was performed with the following PCR profile: 3 min at 94 °C (1 cycle); 35 cycles of 1 min at

Table 1. Details of primers derived from human prostate specific antigen gene of PSA

Primer	Position on gene	Length	Sequence(5-3)
F	555-572	18 nt	TCAGCCTCTGGGTGCCAG
B	979-996	18 nt	TTCCCCTTTAGTAAAGCA
		20 nt	GTGTCACCATGTGGGTCCCG
B3	902-921	20 nt	GAGCGGGGACCTGGTGTGGG
FIP (F1c and F2)	755-776 and 714-735	48 nt	TTGACAGTCAGGGCTGGCTCCCTTTTTGGATTGGTGAGAGGGGCCATG
BIP (B1c and B2)	797-818 and 759-780	48 nt	AACCCAGCACCCCAGCCCAGACTTTTGAGGGGCTGGGGGTATGGGCTT

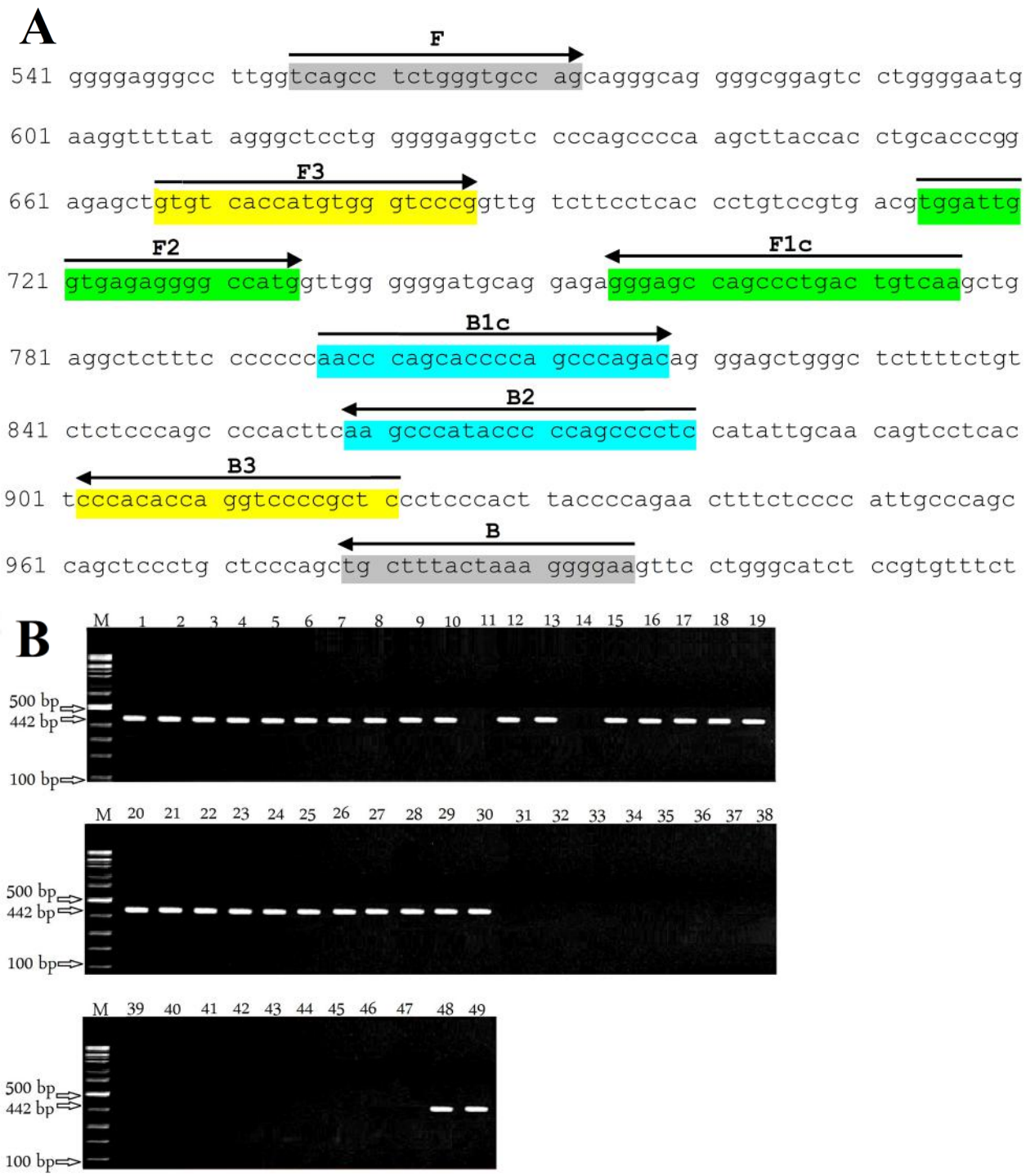

Figure 1. Position of oligonucleotide primers used for detection of the PSA (**A**). Results of electrophoresis of RT-PCR products (**B**). M, DNA size marker (100 bp); lanes 1-15 (localized cancer samples); lanes 16-30 (metastatic cancer samples); lanes 17-45 (BPH samples); lane 46 (negative control: female); lane 47 (negative control: male); lane 48 (positive control: PC3) and lane 49 (positive control: LNCaP).

94 °C, 1 min at 54 °C, 1 min at 72 °C and 10 min at 72 °C for final extension. RT-PCR products were visualized by staining with ethidium bromide after electrophoresis on 1% agarose gel. Finally, using a UV transilluminator (GELDOC 2000, Bio-Rad, USA) a photo of each gel containing RT-PCR fragments (expected size 442 bp) was produced.

Optimization of reaction and RT-LAMP assay

The mentioned positive control was employed to examine and optimize the impacts of temperature, time, dNTP, betaine, and Bst DNA polymerase concentrations. Afterwards, the isolated cDNA underwent an RT-LAMP reaction. The cDNA was incubated at 95 °C for 3 min and chilled on ice for 3 min. 3 µl of cDNA as the template in the RT-LAMP total volume (25 µl) contained 20 mM Tris-HCl (pH 8.8), 10 mM (NH4)-2SO4, 10 mM KCl, 2 mM Betaine (Sigma-Aldrich, Oakville, Ontario, Canada), 0.1% Triton X-100, 10 mM each of dNTP, 0.8 µM each of FIP and BIP, 0.2 µM each of F3 and B3 and 4 U of Bst DNA polymerase (New England Biolabs Inc.). After incubating the mixture in a water bath at 62 °C for 60 min, segregation of the products was conducted using electrophoresis on a 1.5% agarose gel. Also, prior to the amplification, 1 µl of the GeneFinderTM (Biov. Bio. Xiamen, China), was added to the RT-LAMP master mix to provide a visual detection of the RT-LAMP products. The color change occurring in the tubes (positive reaction) from red (prior to amplification) to green (post-amplification) was monitored by visual observation.

Sensitivity of the assay

Comparison of the sensitivity of the detection methods was carried out using the cDNA of the positive control (a tenfold dilution from 1×10^{10} to 1×10^{1}).

RESULTS:

PSA was detected in samples and positive controls by RT-PCR and the predicted DNA fragment (304 bp) was observed on agarose gel (**Figure 1B**). 28 patients (out of 30 prostate cancer patients) were suffering from localized and the rest from metastatic prostate cancer. No sign of PSA was observed by RT-PCR in the samples derived from healthy individuals and those with BPH. Nevertheless, as **Table 2** shows, metastatic (100%) and localized prostate cancer patients (86.6%) and positive controls had PSA transcripts. In brief, both the control subjects and BPH were reported negative by RT-PCR. The effects of temperature and time, as well as the con-

Table 2. Correlation between PSA, clinical and pathological results

	Total	Positive		Negative	
Classification of Patients	No	No	%	No	%
Localized cancer	15	13	86.6	2	13.4
Metastatic cancer	15	15	100	0	0
Benign prostate hyperplasia (BPH)	15	0	0	15	100
Negative control (men)	1	0	0	1	100
Negative control (women)	1	0	0	1	100
Positive control (PC3)	1	1	100	0	0
Positive control (LNCaP)	1	1	100	0	0
Total	49	30	61.22	19	38.78

centrations of dNTP, Bst DNA polymerase and Betaine were examined. Temperature range was considered between 58-68 °C. The results showed that amplification occurred at 60 to 64-65 °C (**Figure 2A**). Moreover, the minimum time for completion of the reaction was 40 min (**Figure 2B**). To test the effects of dNTP concentration on LAMP reaction, final concentration of 1 mM to 10 mM was prepared. The results showed that at 7 mM to10 mM, ladder-like DNA fragments were clearly visible (**Figure 2C**). Different concentrations (1 U to 10 U) of Bst DNA polymerase were used to select minimum concentrations with good performance. With a low concentration of enzyme (3U), poor amplification of DNA was observed, but by increasing the enzyme concentration to 4 U, and further up until 10 U, amplification was considerably improved (**Figure 2D**). The effect of a Betaine concentration from 5 mM to 1000 mM was examined. When the concentration of Betaine was increased from 5 mM to 75 mM, the intensity of the amplified products increased but, no visible products were detected when the concentration was increased to 200 mM (**Figure 2E**).

After achieving optimal conditions and testing 47 samples via RT-LAMP assay, a large number of DNA fragments were observed in a ladder-like pattern by electrophoresing the amplicons on 1.5% agarose gel (**Figure 3A**). It was possible to see the amplicons with the naked eye by detecting color changes in the solutions with the help of various visual dyes. Positive (green) and negative (red) samples could be clearly

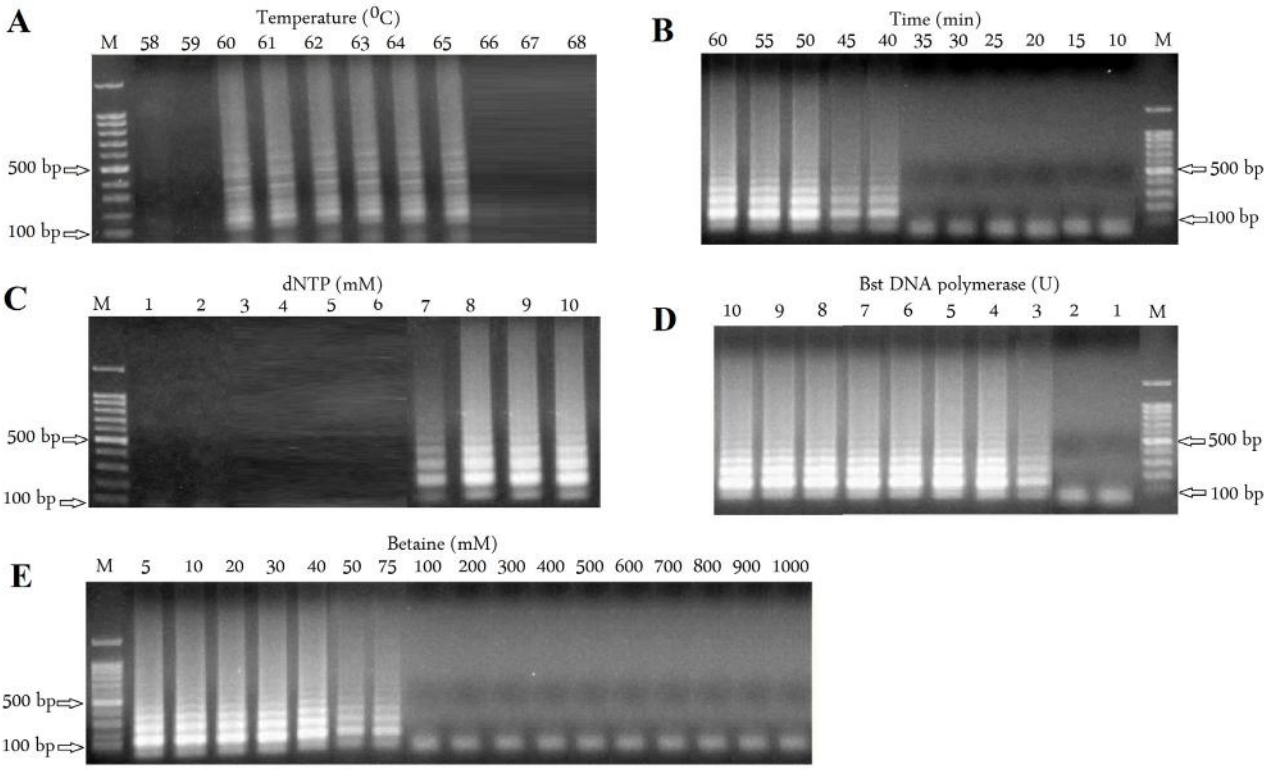

Figure 2. Results of optimization of RT-LAMP reaction. Effects of temperature (**A**), effects of the reaction time (**B**), effects of dNTP concentration (**C**), effects of Bst DNA polymerase concentrations (**D**), effects of Betaine concentrations (**E**) and DNA size marker (100 bp) (M).

and successfully distinguished from each other using GeneFinder™.

RT-LAMP assay yielded products with even lower concentration of sap dilutions (1×10^2 or more), whilst RT-PCR required a higher concentration (1×10^5 or more) (**Figure 3B**). RT-LAMP assay had a higher sensitivity for the detection of PSA in comparison with RT-PCR (1000-fold).

DISCUSSION:

The serum PSA screening test has revolutionized the early detection of CaP, but the high false-positive rate of the PSA test has led to unnecessary biopsies, a matter that has caused concern[21]. With the enormous increase in the rate of CaP incidence, the review of current practices for screening, diagnosis, and staging have been highly investigated[22.] The majority of new CaP cases are clinically localized without obvious metastases. However, 30-40% patients show biochemical recurrence after treatment of localized disease[23]. It is apparent that accurate detection, staging, and prognostic tools are needed, since many CaP patients are not cured by local therapies due to the existence of occult micro-metastases. Diverse technological approaches have been used to evaluate the presence of circulating PSA-expressing cells (CPECs) or tumor DNA in the peripheral blood of CaP patients. Analysis of CPECs in the blood of CaP patients has been performed through cytokeratin-immunomagnetic isolation[24]. Qualitative characterization of CPECs can be achieved through magnetic cell sorting and immunocytochemistry[25]. Additional studies on CPECs by density gradient centrifugation and immunomagnetic bead selection of epithelial cells from peripheral blood have been carried out by several groups[26,27]. Other techniques for CPEC isolation such as using ammonium chloride and distilled water erythrocyte lysis have been tested; however, isolation through density gradient separation and Ber-EP4 immunocapture are more sensitive and efficient[28]. Analysis through flow cytometry on immunomagnetic-enriched epithelial cells was also investigated; however, sensitivity and specificity were limited because of the nature and technique of specimen collection[29]. Studies on methylation-specific PCR targeting promoter hypermethylation of the glutathione S-transferase P1 gene for the possible molecular detection of CPECs have been performed on various human bodily fluids, including plasma, serum, urine, and blood. Glutathione S-transferase P1 promoter hypermethylation was found in 90% of tumors, 72% of plasma or serum samples, and 76% of urine specimens[30]. However, the nature and origin of the circulating DNA tested by methylation-specific PCR has not been defined. The ability to detect small numbers of CPECs by sensitive RT-PCR-based methods, despite current limitations, represents the potential of molecular technology in aiding the detection and staging of CaP[1,4,6].

Peripheral blood-based RT-LAMP assay was applied to investigate PSA in Iranian prostate cancer patients compared to BPH patients in the present research; healthy individuals were also entered as control subjects. Similar results were obtained in a preliminary evaluation by RT-PCR assay. Here, the products amplified via RT-LAMP could be readily visualized with the help of color indicators without any additional staining systems involving toxic materials. Even though RT-LAMP and RT-PCR techniques had enough potential to differentiate and detect infected samples accurately, RT-LAMP proved to be more useful after considering overall time, safety, cost and user friendliness (**Table 3**). Contrary to the proposed approach displaying the advantages of simplicity, user-friendly, and cost-effectiveness, any other methods for PSA detection, require professional personnel to work in labs equipped with costly molecular instruments[17]. Furthermore, no thermocyclers and gel electrophoresis were needed for accomplishing LAMP assay as it could be easily conducted in a water bath or through temperature block[19].

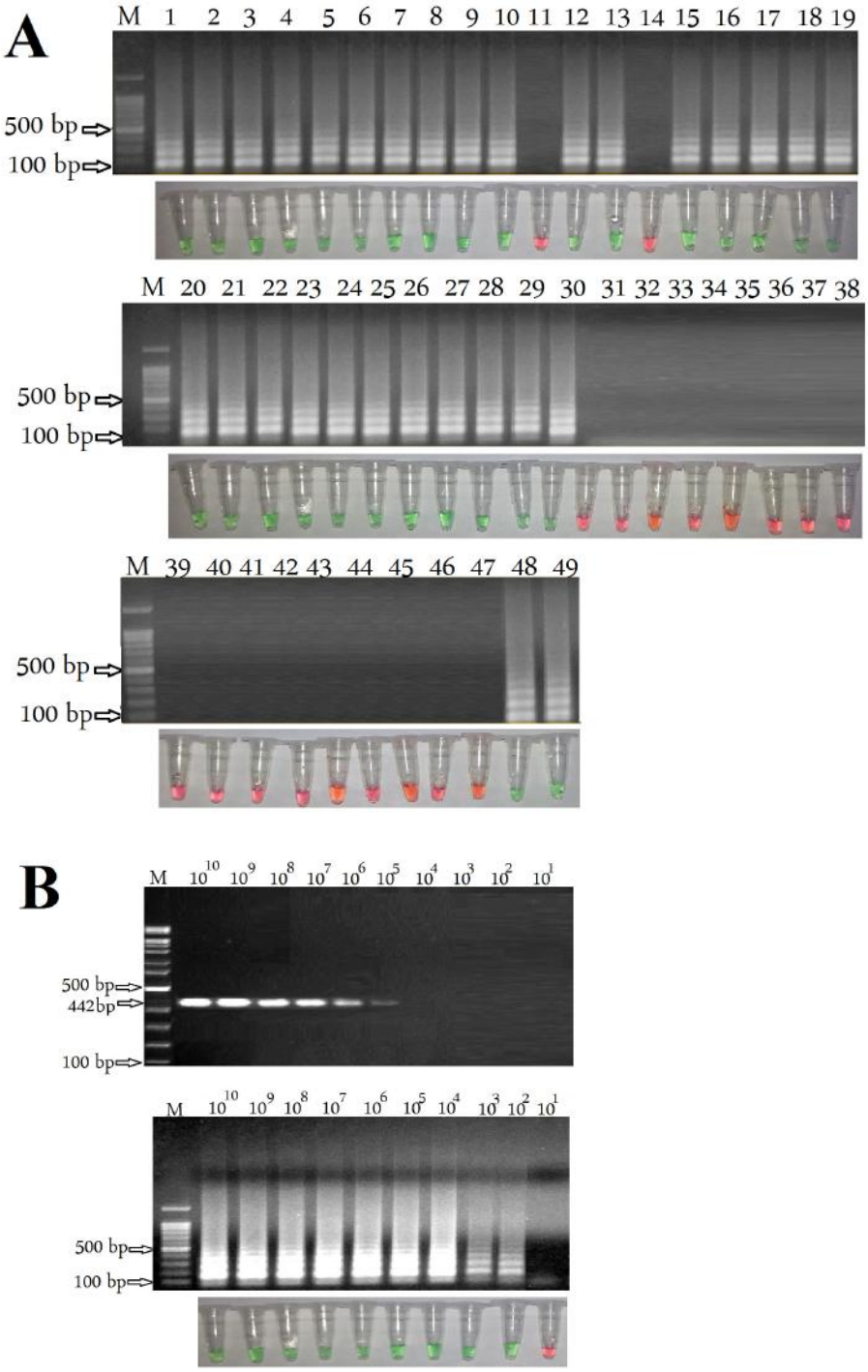

Figure 3. Results of RT-LAMP assay by electrophoresis of products and reaction tube (**A**) and comparison of sensitivity using a ten dilution series (**B**). M, DNA size marker (100 bp); lanes 1-15, (localized cancer samples); lanes 16-30, (metastatic cancer samples); lanes 17-45, (BPH samples); lane 46, (negative control: female); lane 47, (negative control: male); lane 48, (positive control: PC3) and lane 49, (positive control: LNCaP).

Table 3. Comparison of RT-PCR and RT-LAMP assays

Assay	amplification time	Detection method	Safety	Need to UV ray	Need to detect instruments	Cost	User Friendly	Sensitivity
RT-PCR	180 min	Gel electrophoresis	No	Yes	Yes	High	High	10^5
RT-LAMP	30 min	Visual/Gel electrophoresis	Yes/No	No/Yes	No/Yes	Low	Very high	10^2

Generally, the need for additional staining for pursuing post-amplification processes can be obviated by making easier and quicker visual detections via RT-LAMP in-tube color indicator[16]. It is worth noting that in contrast to colorimetric-based methods, GeneFinderTM dye-based assays are more advantageous due to their involving addition prior to amplification; therefore there is no need to open the assayed samples, decreasing the risk of sample contamination[17,18].

CONCLUSION:

In this research, RT-LAMP positive amplicons were observed with the naked eye by adding fluorescent dye to the reaction tubes. Conclusively, cross-contamination risks would be reduced by using and adding GeneFinderTM dye to the reaction mixture before amplification without the need to open the assayed samples. Hence, RT-LAMP assay has several remarkable advantages over any other colorimetric-based methods and can serve as a suitable approach not only to the laboratory detection of PSA, but also to field diagnoses of molecular epidemiology research.

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CONFLICTS OF INTEREST:

All authors declared no conflict of interest.

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