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Development of a loop-mediated isothermal amplification of DNA by using florescent probe in detection of salmonella

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Abstract

Salmonellas are a group of Entrobacteriace that cause Infection disease in human and animals .Thyphoid, Bacteriemi ,Entrocolit and Salmonelose are cause by this bacteria turned to be a major health problem in developing countries ,there for quick , accurate ,reliable and on time diagnosis seem to be vital to prevent it from spreading widely and become epidemic . Different method are used to diagnosing of salmonellas, all of this method require long time to diagnose, numerous bacteria ,expensive and specific lab equipment and expert lab personal. In this research, we used lamp (loop mediated Isothermal amplification of DNA) method with florescent probe to diagnosis five types of salmonella and compare with PCR and lamp. Then optimized this method by the time, probe concentration, and temperature and optimum condition was obtained at temperature on LAMP using fluorescent probe at 37°C, time at 0.2 and concentration of pobe at 4%. According to the obtained results rapid ,accurate ,economic, easy to use , specific and sensitive are the most important factors to evaluate and assay to detect of this method (LAMP by using florescent probe) have extremely all of them that was be used in detection of pathogenic viruses, bactereria, parasites and other medical diagnosis laboratories ,lea gal medical ,agricultural and research filed that takes tie time lees than PCR and LAMP ,this method is independent to cycles temperature and thermo cycling that replacement with thermo block and color detection is more accurate and facilitate than turbidity detection in LAMP.

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Introduction

Salmonella causes three kinds of diseases in human including Typhoid fever, Bacterimi and Enterocolitis, each of which produce several side-effects such as fever, headache, vomiting, diarrhea, Cholecystitis, enlargement of liver size, splenomegaly and joint lesions (Brooks *et al*, 2006). With respect to what was mentioned, we can conclude that quick and timely diagnosis of this bacterium in food stuff and suspected people with infection is necessary and is considered the most important step in treatment. Currently, different experimental methods such as blood, urine and stool culture as bacteriology methods are used to find Salmonella and serology and biochemical methods are based on specific antiserum to diagnose the type of bacterium and the degree of antibody in patients' serum. Conducting this test and obtaining results takes a few days (Murray *et al*, 1990, Abhyankar, 2002).

Other methods are electrical techniques and analysis of nucleic acid markers but there are many problems in terms of their specificity and sensitivity such as difficulty, long running time and technical problems (Andrews *et al*, 1995).

PCR method, attracting biological scientists' attention during recent years, is a kind of DNA replication using natural components of DNA replication which replicates DNA in a test tube by electrophoresis gel. Due to high efficiency, variety of products and alleviating the probabilities of personal errors and increasing reliability of results, this method has been extensively applied over recent years. But this method also has some limitations including application of thermal cycles for replication (30 to 40 cycles) and application of thermo-cycler device which is expensive, PCR product clarification and recognition methods, all of which have led to application of this method only in equipped laboratories with experts in this area. In locations where it is not possible to use advanced laboratory equipment and in remote areas where it is required to diagnose microbial agents such as salmonella to prevent from epidemics and harmful complications, we may use this method (Morel, 2005, Karami *et al*, 2002).

Much research was conducted to remove thermo cycler from this method until the idea of creating gene replication methods in low and constant temperatures was formed after more careful and closer studies on gene replication process into living cells in the same temperature and temperatures much lower than 72˚C. These methods are called Isothermal DNA Amplification methods including a broad area of methods for gene replication without needing thermo cycler. Among these methods we may refer to LAMP method which is Loop-Mediated Iso thermal Amplification of DNA. This method was introduced by Notomi *et.al* (2000) with more important characteristics than those of prior methods in which DNA replicates with high specificity, efficiency and speed in Isothermal conditions. This method can recognize DNA in less than 6 copies of reaction mixture (Notomi *et al*, 2000, Wang *et al*, 2008).

It is clear that LAMP has a number of limitations such as complexity of designing multiple primers for replication of new gene area and selection of suitable regions in gene sequence for suitable and efficient designing of primers and creating complex product from replication (Demidov, 2002).

Since identification of turbidity is relative and achromatic in determining positive responses without sufficient precision and sensitivity, addition of fluorescent probe as a marked material reflecting our positive sample with a fluorescent color is useful for interpretation of results (Iwamato *et al*, 2003, Mori *et al*, 2006).

In this study, we employed five various strains of salmonella (Salmonella Typhi, Salmonella paratyphi B, Salmonella paratyphi C, Staphilococose aureus). We used lamp (loop mediated Isothermal amplification of DNA) with florescent probe to diagnosis of salmonella and compare with PCR and then the time, probe concentration, and temperature

of this method were optimized and optimum conditions was obtained at 37°C and 60 min by 2.5l concentration of probe.

Materials and methods

Materials

In this study, we used five different strains of salmonella (Salmonella Typhi, Salmonella paratyphi B, Salmonella paratyphi C, Staphilococose aureus). In order to standardize the test, standard salmonella strains were supplied from reference laboratory of health department.

Chemical Material used in this study were purchased from Merck Company, nucleotide buffer and Taq polymerase was provided by Iranian companies and BST polymerase and probes were purchased from BioRad .Master cycler made by Eppendrof for thermo-cycles in PCR and Thermo Block by Kiagene Co. for conducting LAMP and LAMP with fluorescent probe, a small horizontal electrophoresis device by Paya Pajoohesh for examination of gel from Uvidoc device by UvTec Co., 100 bp Molecular marker from Fermentaz Co. and Nanodrop Spectrophotometer for investigating photo-absorption of fluorescent material in expected wavelengths were used. We used primers designed by Meada *et al* to conduct PCR (Karami and Ahmadi, 2006, Karami *et al*, 2007). Also; probes were designed by Karami for this method.

Methods

Bacterial strain and DNA extraction

At first 50 µl from each bacterial sample was taken and cultured in LB culture than incubated at 37°c for 18-24 hr and after this time bacteria were grown.

Each bacterial sample were cultured In 1.5 cc of LB Medium and incubated in 37 °c for 18-24 hr. after this time we extracted DNA of them by Fenul-chloroform standard method (Kochl *et al*, 2005). In this method we qualified the extract DNA with Gel electrophoresis 1% and compared them by DNA standard bond .So we can isolate the DNA extract concentration.

Polymerase chain reaction (PCR)

In this method, after extracting Genome, primers designing step is important. Primers were designed based on InvA (InvasionA) gene which is responsible for bacterial invasion to epithelial cells in intestines and increases pathogenic effects of bacteria (Table 1). Then in order to perform PCR, 1µl of extracted genome samples was added to the tube containing compounds in Table 1 and the samples were located in Master Cycler device and PCR was performed according to the plan in the first step, the first step includes 1 minute at 96˚c and then 30 cycles including 3 phases of 94˚ in 30 seconds, 57˚ in one minute, 72˚ in one minute and finally 72˚ for five minutes. Finally, 5 to 10 µl of the product was mixed with 1 to 2 µl of specific sampling buffer (GLB) and run for 45 minutes in Agarose gel 1% with voltage 80 mv. After staining the gel with Etidium Bromide, we took pictures of the gel with UV device (Table 2).

Loop-Mediated Isothermal Amplification (LAMP)

In order to conduct LAMP test, 4 primers were used which were designed by Maeda *et.al* (Mead, 1991, Natsu *et al*, 2008, Amaresh *et al*, 2012, Yukiko Hara *et al*, 2005, King *et al*, 2013) for molecular diagnosis of salmonella based on InvA gene (Figure 1), the sequence of which is presented (Table3).

In order to conduct LAMP, 1 µl of genome samples was added to tubes containing compounds in table 4 and the samples were put in Thermo Block device. Then LAMP was performed based on the following program. The first step lasted 60 minutes at 65˚c and the second step was 10 minutes at 82˚c. After completion of the test, 5-10 µl of the product was mixed with 1-2 µl of sampling buffer (GLB) and the test was run in Agarose gel 2% with voltage 85 for 45 minutes. Then the gel was stained with a solution containing Etidium bromide and the sample with buffer was photographed and examined using UV device (Table 4).

LAMP using fluorescent probe

In this method, four primers (two Forward and 2

Reverse primers) designed by Meada et.al (Mead, 1991, Mori *et al*, 2001, Chou *et al*, 2011, Siyi and Beilei, 2010, Zhidi *et al*, 2011) for molecular diagnosis of salmonella and two probes designed by Karami was used (Table5). 1 µl of extracted genome samples was added to tubes containing the compound (Table 6) and were put in Thermo Block device. According to the plan (Figure 2), in the first step the reaction was conducted at 65˚c for 60 minutes and then at 62˚c for 10 minutes. After completion of the program, photo-absorption of each sample was measured by Nano drop spectrophotometer.

Results and discussion

Polymerase chain reaction (PCR)

After electrophoresis and gel staining using a solution containing Etidium bromide and washing by buffer, the gel was photographed by UV device. Investigating the samples using two pairs of primers designed based on InvA gene and conducting PCR on them, gave us two bands of 373 pb and 258 pb which were observed in all salmonella. In PCR, only one band is there for each pair of primers. For primers S_3 and S_4, band 258bp and for primers S_{13} and S_{12}, band 373bp was noticed (Figure 3).

Table 1. PCR Primers Sequence.

Serial	Probe	Sequence $(5'-3')$	Size (bp)	Size of PCR product (bp)
1	S12	$5'-GTATTGTTGATTAATGAGATCCG-3'$	23	373
2	S13	$5'-ATATTACGCACGGAAACACGTT-3'$	22	

Table 2. PCR Master Mix (25 µl).

Concentration	Master Mix (25 µl)	Name	Serial
-	14 µl	D.D.W	1
1x	2.5 µl	Buffer 10x	2
1mM	0.5 µl	$Mgcl_2$(50mM)	3
0.1mM	0.5 µl	dNTP (5Mm)	4
2.5 Unite	0.5 µl	Taq Poly merase	5
20 P .mol	3 µl	F.Primer	6
20 p.mol	3 µl	R.Primer	7
75 nano gram	1 µl	DNA	8
-	25 µl	Total	9

Table 3. Primer of LAMP Sequence.

Printer	Type	Length	Sequence
FIP	Forward inner $(5^{\circ}-FIC-TTTT-F2-3^{\circ})$	46nt(F1C, 22nt, F2,20nt)	$5'-GAGGGGTGGTACTGATCGA$ $TAGTTTTTCAACGTTTCCTGGGG-3'$
BIP	Backward inner $(5^{\circ}-BIC-AAAA-B2-3^{\circ})$	45nt(B1C, 21nt, B2,20nt)	$5'-CCGGTGAAATTATCGCCACAC$ $AAAACCCACC GCCAGG-3'$
F3	Forward outer	22nt	$5'-GGGGATATTG\ GTGTTTATGG\ GG-3'$
B3	Backward outer	20nt	$5'-AACGATAAACTGGACCAGGG-3'$

Loop-Mediated Isothermal Amplification (LAMP)

After electrophoresis on gel Agarose 2% and gel staining by a solution containing Etidium bromide, the sample was photographed by UV device and it was determined that several scalar bands with sizes larger than 241bp were obtained and no band was visible in negative control without DNA (Figure 4).

Turbidity in LAMP

Clarification of final results is possible according to

turbidity of reaction environment due to release of pyrophosphate from dNTP and its combination with magnesium ions. The turbidity is a method for identifying the positive sample (Figure 5).

LAMP using fluorescent probe

After performing optimization reactions over time, probe concentration, the effect of light and temperature, and conducting reactions in optimal conditions of photo-absorption of samples were measured using nanodrop spectrophotometer device in wavelengths zero to 700 nm. With respect to the type of probes applied here, photo-absorption of samples in expected wavelengths was investigated.

Table 4. Master Mix of LAMP.

Concentration	Master Mix (25 µl)	Name	Serial
-	11.5 µl	D.D.W	1
0.1mM	2.5 µl	Buffer 10x	2
0.1mM	0.5 µl	dNTP (5Mm)	3
8 mM	0.5 µl	Bst Poly Merase	4
20 P .mol	2 µl	FIP Primer	5
20 P .mol	2 µl	BIP Primer	6
5 P .mol	0.5 µl	F3 Primer	7
5 P .mol	0.5 µl	B3 Primer	8
0.4 mM	2 µl	Betaine	9
75 nano gram	3 µl	DNA	10
	25 µl	Total	11

Table 5. Sequence of probe used in LAMP fluorescent.

Serial	Probe	Sequence $(5'-3')$	Size (bp)	
1	Probe A	[5]GAGGAAAGAGCGTGGTAATTAAC	23	$5'-FAM,3'-TAMRA$
2	Probe B	GGGCAATTCGTTATTGGCGATAG	23	$5'-FAM,3'-TAMRA$

Table 6. Master Mix of LAMP by using florescent probe.

Concentration	Master Mix (25 µl)	Name	Serial
-	6.5 µl	D.D.W	1
0.1mM	2.5 µl	Buffer 10x	2
0.1mM	0.5 µl	dNTP (5Mm)	3
8 mM	0.5 µl	Bst Poly Merase	4
20 P .mol	2 µl	FIP Primer	5
20 P .mol	2 µl	BIP Primer	6
5 P .mol	0.5 µl	B3 Primer	7
5 P .mol	0.5 µl	F3 Primer	8
25 P .mol	2.5 µl	Probe A	9
25 P .mol	2.5 µl	Probe B	10
0.4 mM	2 µl	Betaine	11
75 nano gram	3 µl	DNA	12
	25 µl	Total	13

Optimization of the LAMP using fluorescent probe

In order to have the best results we do some optimization on time, temperature and probe concentration.

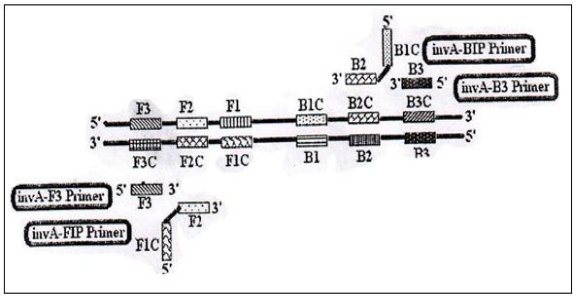

Fig. 1. Primer and Probe of LAMP Structure.

Influence of time on LAMP using fluorescent probe

In order to optimize time and obtain a suitable time to have the maximum replication, the reaction was run in two different durations of 30 and 60 minutes. The results suggest that we have the maximum products in 60 minutes.

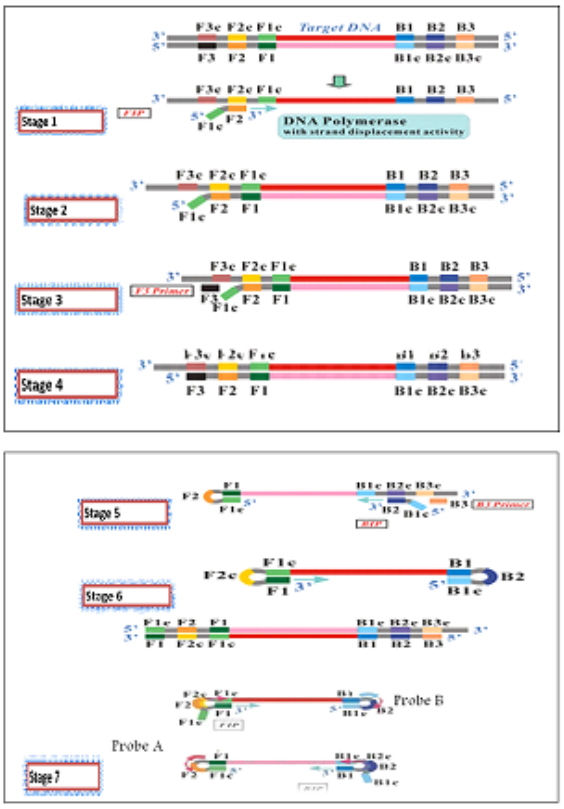

Fig. 2. Process of LAMP fluorescent.

Influence of temperature on LAMP using fluorescent probe

In order to optimize temperature and obtain a suitable temperature to have the maximum replication, the reaction was run in two different 62 °c and, 65 °c temperature. So we have the maximum products in 65 °c.

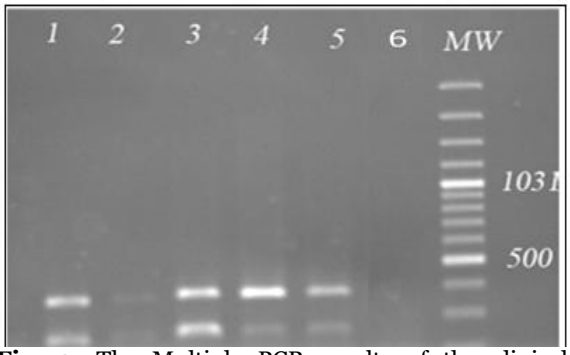

Fig. 3. The Multiple PCR results of the clinical templates [lane1: Salmonella typhi, lane2: Salmonella paratyphi A, lane3: Salmonella paratyphi B, lane4: Salmonella paratyphi C, lane5: Salmonella enteritidis, lane 6: Staphilococose aureus (Neg control), lane 7: Molecular marker.

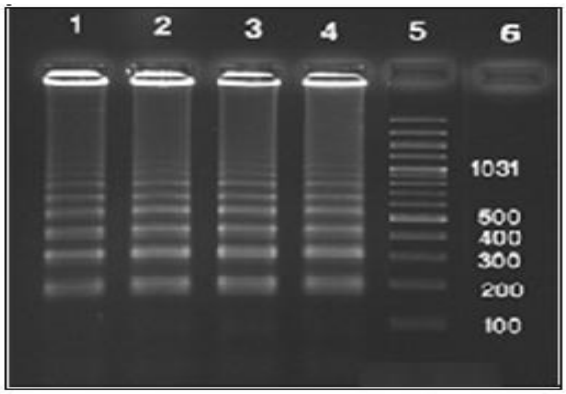

Fig. 4. The Multiple LAMP results of the clinical templates [lane1: Salmonella typhi, lane2: Salmonella paratyphi A, lane3: Salmonella paratyphi B, Lane4: Salmonella paratyphi C. Lane5: Salmonella enteritidis, Lane6: Staphilococose aureus (Neg control).

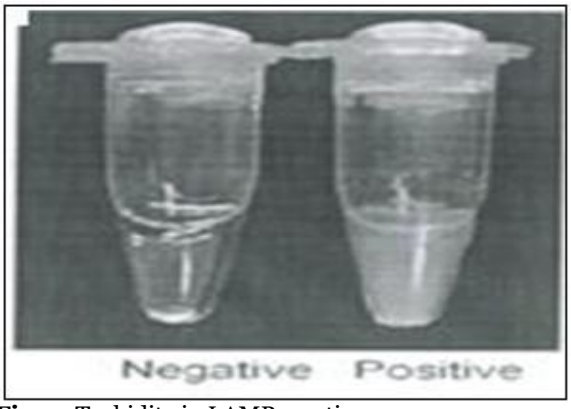

Fig. 5. Turbidity in LAMP reaction.

Influence of probes concentration in LAMP using fluorescent probe

Concentration of probes used during replication is very important. Therefore, for optimization, the reaction was performed in three concentrations 2, 2.5 and 3 µl of probe. After completing the cited reactions it was recognized that 2.5 µl of probe is an optimal concentration for the maximum photo absorption which is consistent with the maximum replication.

Conclusion

Salmonellosis is responsible for large numbers of infections in both humans and animals.. PCR method is used to diagnose salmonella and separate it from other Entrobactriace, which needs more than three hours. LAMP method was applied which has much better characteristics than previous methods in single temperature conditions during which DNA was replicated in a shorter time with higher efficiency and specificity (using 4 primers). Since turbidity is relative and achromatic in determining positive samples without sufficient precision and sensitivity, application of a probe as a marked material to reflect the positive sample as a fluorescent color helps us in interpreting the results and conducting the test with more reliable results so that positive sample identification will not need scientific and specialty information.

In addition to 4 primers (two outer and two inner), we used 2 probes to achieve the cited goals. After the necessary modifications in time, temperature and concentration of the probe and the effect of testing light at 65˚c, the presence of light and concentration of 2.5 µl over 60 minutes, photo absorption of samples was investigated with nanodrop spectrophotometer in wavelengths zero to 700 nm. Use of two fluorescent probes for visual inspection of LAMP results was superior technique with no gel electrophoresis and staining required. This two fluorescent probe we used (3´-TAMARA, FAM-´5), the maximum photo absorption was obtained on the expected points. (430, 500 nm) during these reactions we achieved a more economical, and simple method with higher sensitivity and specificity which is very quicker (three times), more precise (to 10 times) and cheaper (to 10 times) than PCR method with a broader application in medical laboratories and even small mobile labs to study epidemics and molecular diagnosis of biological agents.

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