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IMMOBILIZATION WITH METAL HYDROXIDES AS A MEAN TO CONCENTRATE FOOD PATHOGENS FOR INCREASING SENSITIVITY OF PCR BASED DETECTION METHOD

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SUMMARY

Polymerase chain reaction (PCR) based bacterial detection without enrichment step could be applicable by concentrating bacteria from food matrix by metal hydroxide. The false positive from exogenous DNA of dead cells could be eliminated by DNase I treatment (15 U, 1 hour, 37°C), one step before DNA extraction. DNase I facilitated for DNA removal DNA from dead cells and had no detectable damaging effect signal on the DNA presented in live/intact cells. Double-stranded DNA dye SYBR green I was used in the real time PCR to quantify the amplicon production. In immobilization process, bacterial recoveries ranged from 93.6 to 99.3% for both metal hydroxides compared to 31.5 - 54.70% with no immobilization. When seeded in 10 ml of whole milk, sample volume was reduced 50 - fold after immobilization by zirconium hydroxide. The detection limits for *E. coli* O157:H7 (384 bp), *L. monocytogenes* (482 bp), *E. coli* wild type (580 bp) was $5x10^1$ cells and of *S. typhimurium* (685 bp) was 5×10^2 cells in 10 ml of whole milk by real-time PCR analysis.

Keywords: *Bacterial recoveries, DNase I, immobilization, real-time PCR, SYBR green I*

INTRODUCTION

Food-borne pathogens such as *Salmonella*, *Escherichia coli*, and *Listeria monocytogenes* are among the most serious threats to human health. A large number of rapid methods have been developed for food-borne hazard utilizing the optical, electrochemical, biochemical and physical properties of microorganisms (Hobson *et al.*, 1995). In general, individual method suffers from various disadvantages that include a lengthy analysis time, high cost of instrumentation and lack of sensitivity. Moreover, pathogen contamination is low concentration so detection includes lengthy culture enrichment steps to increase target bacterial numbers before identification using standard cultural procedures are required. Polymerase chain reaction (PCR) technique is high sensitive but detection limit is still high and requires lengthy enrichment steps. The inability of the PCR method to distinguish dead and viable pathogen cells is another draw back of the method.

Many reports indicated that rapid molecular methods could be improved if the bacteria were separated, concentrated, and purified from the food matrix before detection. Immobilization of bacterial cells with metal hydroxides was reported by Ibrahim *et al.* (1985) for bacterial detection by solid-phase immunoassay. Lucore *et al.* (2000) reported the use of bacterial immobilization with metal hydroxides for the concentration and purification of bacterial from none fat dry milk (NFDM) with reverse transcription (RT)-PCR resulting in high amplification of target mRNA that are short-lived instead of DNA thus reducing the ability of detecting dead cells. However, the use of highly labile bacterial mRNA molecules as template and the multi-step nature of these reactions invariably make them lengthier, costlier and less sensitive compared to DNA-based amplification method (McKllip *et al.*, 1999). In this study we apply DNase I treated DNA (DTD)-PCR to detect bacteria immobilized by metal hydroxide for developing a low cost, sensitive and reliable method to detect and quantify living bacterial pathogens.

MATERIALS AND METHODS

Bacterial strains

Salmonella typhimurium ATCC 13311 was obtained from BIOTEC, NASTDA, Thailand.

Escherichia coli O157:H7, *Listeria monocytogenes,* and *Escherichia coli* wild type were available at Bioprocess lab of Asian Institute of Technology (AIT), Thailand. They were grown overnight at 37°C for 16 hours in Tryptone soya broth yeast extract (TSBYE) medium (Difco Laboratories, Detroit, MI, USA) until used in recovery experiment. Serial dilutions of bacteria were done in 0.9% NaCl (steriled saline) and spread on Nutrition agar (HIMEDIA) to determine the percentage recovery.

Metal hydroxide preparation

For zirconium hydroxide, 40 ml volume of distilled water was added to 2.0 g of zirconium (IV) chloride (Aldrich Chemical Co., Milwaukee, Wis.). For titanous hydroxide, 0.324 M solution was prepared by the addition of 26.7 ml of distilled water to 13.3 ml of titanium (III) chloride 15% (Aldrich Chemical Co.). Ammonium hydroxide (5 M) solution was added drop wise with continuous agitation until pH 7.0 ± 0.2. Each metal hydroxide solution was then washed three times with 200 ml of sterile saline solution to remove excess ammonium ions. During washing process, the hydroxide was mixed gently with the sterile saline solution and allowed to settle over a 10 min period, and then approximately 40% of the top phase (consisting of saline solution and debris) was decanted. The final volume of each hydroxide was between 200 ml and 250 ml. The hydroxide solutions were stored in the dark until required for bacterial immobilization.

Preparation of aliquots of cell suspensions

Bacterial strains were cultured separately in 10 ml of TSBYE broth and incubated at 37°C for 16 hours. Cells were harvested in separate eppendorf tubes by centrifugation at 10,000 rpm for 5 min. Cells were washed twice with 1 ml of 0.9% NaCl and resuspended in 300 µl of 0.9% NaCl. Cells were counted by haemocytometer after diluting in sterilized 0.9% NaCl for 10,000 times. Cell suspensions were placed on ice after counting. Aliquots of cell suspensions with desired concentrations were made for each experiment.

DNA isolation

Aliquots of bacterial sample preparations were immobilized with metal hydroxide, centrifugation at 10,000 rpm for 5 min at 7°C. Cells in the metal hydroxide pellets were lysed by diluted 1:10 with 0.5 ml of 1 - 2% Triton X-100 (1% final concentration) and vortexed. Suspension was heated in boiling water (100°C) for 10 min to release DNA. Samples were cooled to room temperature. Equal volume of chloroform (0.5 ml) was added to the suspension and mixed well. Suspension phases were separated by centrifugation at 10,000 rpm for 10 min, at room temperature. The upper phase containing was added equal volume of 100% isopropanol (0.5 ml), mixed gently by inverting the tube. DNA was collected by centrifugation at 12, 000 rpm at 7°C for 10 min. The DNA pellet was washed with 1 ml of 70% ethanol followed by centrifugation at 10,000 rpm for 5 min at 7°C. DNA was dried and then resuspended in 20 µl TE buffer. DNA solution was stored at −20°C until real time PCR analysis.

PCR primers

The primers used in this study are listed in Table 1. All the oligonucleotide primers were synthesized by the Bioservice Unit of BIOTEC, Thailand.

Real time PCR was performed using Hotstar Taq Master mixture (Qiagen) at final concentration of Tag DNA polymerase (1 U/20 µL), $MgCl_2$ (1.5 mM), PCR buffer 1×, dNTPs (200 mM) and 5 µl genomic DNA (variables), 0,5 µl of each primers (25 µM), 2 µL of SYBR green I at final concentration 1/70 000 of stock solution.

Real time PCR amplifications

Amplification and detection of DNA by real-time PCR were performed with Biorad icycler IQ, carried out in 20 µl reaction volume. The amplification profile was 95°C for 600 s (10 min) (initial denaturation), 95°C for 45 s (denaturation), 45°C for 90 s (annealing), 72°C for 75 s (extension), 72°C for 300 s (final extension) and soaked at 4°C.

Gel electrophoresis

Products of real time PCR were analyzed on 2% (w/v) agarose gel in 0.5× TAE buffer staining ethidium bromide (0.5 mg/l) and visualized by UV light.

Standard curve for bacterial strains in real-time PCR assay

This experiment was carried out in order to find out the relationship between threshold cycles (C_T value) Vs. (Log initial cell number for four bacteria strains including Gram positive and Gram negative

such as *S. typhimurium, L. monocytogenes, E. coli* O157:H7, *E. coli* wild type. The serial dilutions were made for 0.5 ml of saline solution to achieve final cell number of 5×10^5, 5×10^4, 5×10^3, 5×10^2, 5×10^1, 5×10^0 then added 0.5 ml of 2% Triton X-100 and vortexed. DNA was extracted by Triton X-100 and dissolved in 20 µl of TE buffer, stored at -20°C.

Evaluate immobilization of various bacterial strains by real time PCR

This experiment was done in order to find out the effect of volume ratio of samples to metal hydroxides on bacterial immobilization efficiency. The bacterial strains were immobilized with zirconium hydroxide (ZiOH) and titanous hydroxide (TiOH) as following: 100 µl of serial dilutions of bacterial strains at final concentrations of 10^6, 10^4, 10^2 cells/ 100 µl were immobilized with 100 µl, 200 µl and 0 µl of metal hydroxide to present a 1:1, 1:2, and 1:0 volume ratio of sample to metal hydroxide, respectively followed by centrifugation at 10, 000 rpm for 5 minutes at 7°C. Obtained pellets were added with 0.5 ml of 1% Triton X-100, vortexed. DNA was extracted. Recovery was calculated as following: Bacterial recovery (%) = (C_T value) × 100 / (C_T value in control tube).

Feasibility of DNase I treatment for PCR based detection

Experiment was carried out to observe the effect of DNase I to heat killed bacteria cells and on the DNA presented in live cells of Gram positive and Gram negative reference organisms.

Table 1. PCR primers used in this study.

Species	Target gene	PCR primers' sequences (5' - 3')	Product size	Reference
E. coli O157:H7	nt 27 - 410 of attaching and effacing A (*eae A)* gene	*eae* F: GAC CCG GCA CAA GCA TAA GC *eae* R: CCA CCT GCA GCA ACA AGA GG	384 bp	Paton, 1998
E. coli wild type	nt 27 - 410 of attaching and effacing A (*eae A)* gene	*eae* F: GAC CCG GCA CAA GCA TAA GC *eae* R: CCA CCT GCA GCA ACA AGA GG	580 bp	Paton , 1998
L. monocytogenes	***Listeriolysin O (hly)*** gene	*Hly* F: TAT ACC ACG GAG ATG CAG TG *Hly* R: GCC GAA GTT TAC ATT CAA GC	482 bp	
S. typhimurium	***invA*** gene	*invA* F: TCT CTA CTT AAC AGT GCT CG *invA* R: TGG TAT AAG TAG ACA GGG CG	685 bp	

Preparation of heat killed cells

In order to get bacterial cells that are not viable, the cells were treated in alcohol freezing chamber then autoclaved at 121°C for 15 min, and pressure 120 kgf/cm^2 to kill the cells. Aliquots of cell suspensions prepared were subjected to DNase I treatment. Growth absence of treated cells was checked on TSBYE broth.

DNase I treatment in discriminating viable and unviable cells immobilized by zirconium hydroxide in food model (1 ml of whole milk)

The conditions in bacterial immobilization for detection of live target organisms and its ability to get rid of DNA that yields from dead cells was tested in this particular experiment. Serial dilutions of both living and heat killed cells of four target organisms were seeded in 900 µl of whole milk to achieve a final concentration of 5×10^6 cells/1 ml of whole milk. Obtained solutions were clarified by 60 µl of 25% (w/v) of sodium citrate with 5 min of shaking by hand at room temperature. Separation step was performed by centrifugation at high speed 10,000 rpm for 10 minutes at 7°C. Pellets were reconstituted in 100 µl of 0.9% saline solution. Obtained solutions were immobilized and subjected to 10 U of DNase I (1 U/1 µl) in 150 µl of reaction buffer. The cell aliquots then were mixed and incubated for one hour at 37 ± 1°C, mixing was

carried out at every 30 min intervals. DNA was extracted by adding 0.5 ml of 1.5% Triton X-100 then amplified by real-time PCR and PCR products were resolved on 2% agarose gel containing ethidium bromide.

Sensitivity of DTD-PCR in detecting zirconium hydroxide immobilized bacteria in food application (10ml of whole milk)

Aliquots of 100 µl of living cells with cell count of 5×10^4, 5×10^3, 5×10^2, 5×10^1, 5×10^0 was mixed with 100 µl of the heat killed cells with cell count of 5×10^4, 5×10^3, 5×10^2, 5×10^1, 5×10^0 respectively then spiked into 9.8 ml of whole milk making to 10 ml of final volume. The samples were clarified with 600 µl of 25% (w/v) of sodium citrate. Centrifugation at high speed 10,000 rpm for 10 minutes at 7°C. The supernatants were taken out. Pellets were reconstituted and transferred to small tubes (1.5 ml volume) by adding 0.9% saline solution up to 0.3 ml and immobilized by 0.3 ml of zirconium hydroxide. The pellets were obtained by centrifugation at 10,000 rpm for 5 minutes at 7°C. The pellets were subjected to 15 U of DNase I (5 U/1 µl) in 150 µl of reaction buffer. Extracted DNA was amplified by real-time PCR and PCR products were resolved on 2% agarose gel containing ethidium bromide.

RESULTS

Standard curves in real -time PCR assay

Double-stranded DNA dye, SYBR Green I is a fluorogenic minor groove binding dye that exhibits little fluorescence when it is in solution but emits a strong fluorescent signal upon binding to double-stranded DNA (Morrison *et al.*, 1998). DNA amplicons from initial cell number of 5 cells of four bacterial strains in this experiment can be detected by real-time PCR after 46 cycles (data was not shown). Cycle threshold (C_T) values were calculated with the standard algorithms. The linear changes for all bacterial strains had a good fit ($R^2 > 0.97$) (Figure 1).

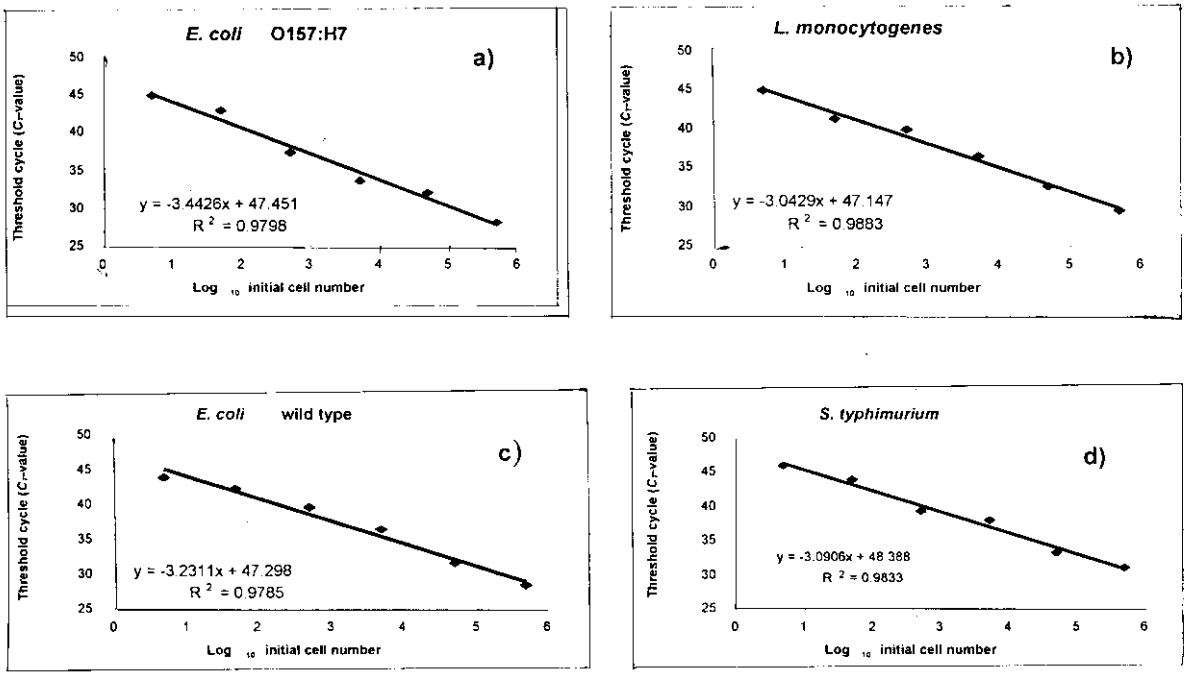

Figure 1. The standard curve of E. *coli* O157:H7 (**a**), *L. monocytogenes* (**b**), *E. coli* wild type (**c**). and *S. typhimurium* (**d**) in real-time PCR assay.

Evaluation of bacterial immobilization with metal hydroxides by real-time PCR

The C_T values obtained from DNA amplificon (data was not shown) were calculated for bacterial recoveries (%) for all tested bacterial strains at different volume ratios 1:1 and 1:2 by both titanous hydroxide and zircornium hydroxide were very high from 93.7% to 99.3%. (Figure 2). The volume ratio of sample to zirconium hydroxide 1:1 was chosen for subsequent experiments in term of saving amount of metal hydroxide. None immobilizarion, the bacterial recoveries were low only 31.5 - 54.7%.

Feasibility of DNase I treatment in discriminating viable and unviable bacterial cells immobilized by zirconium hydroxide in food model (1 ml of whole milk)

This experiment was carried out in order to detect the effect of DNase I treatment to living cells and the effectiveness of DNase I on removing DNA in dead cells. The C_T value or cell counts of live cells subjected to DNase I treatment similar to the untreated one (Table 2). It is clear to indicate that Dnase I treatment has no detectable damaging effect signals on the DNA presented inside the live/intact cells. The treated dead cells gave no DNA amplification in real-time PCR and therefore no detectable signal on agarose gel (Picture was not shown).

Sensitivity of DTD-PCR in detecting metal hydroxide immobilized bacteria in food application (10 ml of whole milk)

The cell counts from untreated DNase I were double as compared to those from one treated DNase I for all tested bacteria strains (Table 3. It is cleared that in the treated samples, the DNA from dead cells was degraded giving no DNA amplification but giving DNA amplification in untreated one. The sensitivity of this experiment would be based on the tubes with DNase I treatment. In DNase I treatment, the detection limit of *E. coli* O157:H7 was 5×10^1 of live cells/10 ml of whole milk (Figure 3.1B) after 42.27 cycles in real-time PCR. The detection limit for *L. monocytogenes* was 5×10^1 of live cells (Figure 3.2B) after 42.89 cycles. *E. coli* wild type was detected at 5×10^1 cells by agarose gel electrophoresis (Figure 3.3B) after 41.51 cycles in real-time PCR. The detection limit of *S. typhimurium* was 5×10^2 of live cells (Figure 3.4B) after 39.76 cycles.

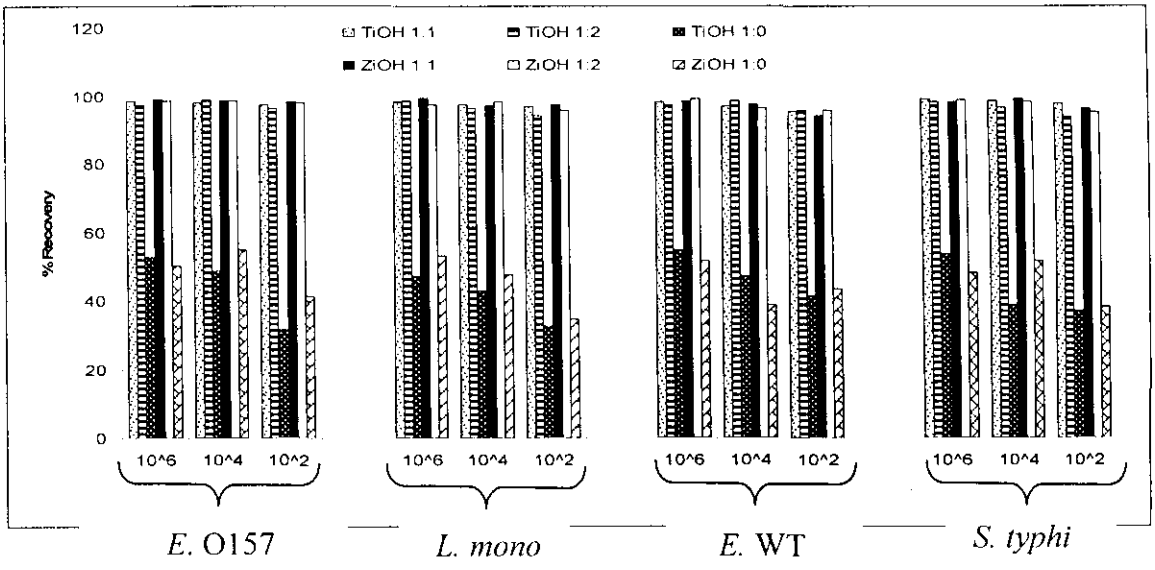

Figure 2. Bacterial recovery (%) at different volume ratios of sample to metal hydroxides in real-time PCR. *E.* O157 = *E. coli* O157:H7, *L. mono* = *L. monocytogenes*, *E.* WT = *E. coli* wild type, *S. typhi* = *S. typhimurium*.

Table 2. DNase I treatment in discriminating viable and unviable bacterial strains immobilized by metal hydroxides in food model (1 ml of whole milk) by real-time PCR.

Bacterial strains	Whole milk							
	Live cells				Dead cells			
	DNase +		DNase -		DNase +		DNase -	
	C_T-value	Count	C_T-value	Count	C_T-value	Count	C_T-value	Count
E. O157	24.53	4,562,398	24.61	4,321,569	na	na	24.73	3,987,562
L. mono	26.97	4,265,124	26.92	4,451,236	na	na	27.04	4,056,125
E. WT	25.93	4,102,108	25.86	4,326,987	na	na	26.09	3,648,975
S. typhi	27.91	4,212,305	27.96	4,056,897	na	na	28.06	3,789,624

Note: na means not analyzed.

Table 3. Sensitivity of DTD-PCR in detecting metal hydroxide immobilized bacteria in food application (10 ml of whole milk).

Organisms	DNase I	L + D (cells)		L + D (cells)		L + D (cells)		L + D (cells)		L + D (cells)	
		$5 \times 10^4 + 5 \times 10^4$		$5 \times 10^3 + 5 \times 10^3$		$5 \times 10^2 + 5 \times 10^2$		$5 \times 10^1 + 5 \times 10^1$		$5 \times 10^0 + 5 \times 10^0$	
		C_T-value	Count	C_T-value	Count	C_T-value	Count	C_T-value	Count	C_T-value	Count
E. O157	+	31.40	45,963	35.21	3,596	38.31	452	42.27	32	na	na
	-	30.41	89,256	33.87	8,781	37.18	965	40.77	87	44.54	7
L. mono	+	32.65	58,236	36.02	4,526	39.52	321	42.89	25	na	na
	-	31.97	97,201	35.00	9,851	38.16	895	41.19	91	45.70	3
E. WT	+	32.39	41,201	35.18	5,621	38.72	451	41.51	62	na	na
	-	31.27	91,256	34.67	8,121	37.63	982	40.91	95	na	na
S. typhi	+	34.21	38,796	37.21	4,132	39.76	618	na	na	na	na
	-	33.17	84,125	36.05	9,853	39.34	845	43.40	41	na	na

DISCUSSION

Standard curve in real time PCR shows that target DNA extracted from 5 cells of four bacterial strains could be detected by both real time and conventional PCR methods. The sensitivity of PCR products given by not taking out supernatants during DNA extraction process. The bacterial recovery here can be assumed as 100% meaning no bacterial cells lost to supernatant. In the case of food, bacteria were clarified and the supernatant of samples need to be taken out by centrifugation before DNA extraction so the recoveries were lower. The idea come out is that sensitivity of DNA amplicon detection can be increased if the percentage of bacterial recovery is increased. This was one of the targets in this study. The percentage recoveries of four bacterial strains to metal hydroxides were high from 93.7% to 99.3% as compared to Berry *et al.* (1997) who used fluorescent staining technique for detection of *E. coli* cells immobilized by hydroxyapatite the recovery was 95%.

DNase I treatment in discriminating viable and unviable bacterial strains was done based on the assumption that when the cells are dead it losses its membrane property of selective permeability and will

facilitate the passive movement of DNase I into the cells. This may accomplish the task of degrading DNA inside the dead cells with intact cellular membrane (Mackey, 1984). In this study huge quantities of killed cells 5×10^6 were dumped into the system (1 ml of whole milk) to convincingly demonstrate the ability of DNase I treatment (10 U, 1 hour, 37°C) to effectively remove the DNA derived from non-living sources of cells taken.

Bacterial detection at low concentration (10 cells, 100 cells) in real-time PCR was possible in later cycles (43.40 - 45.70 cycles). It is better than conventional PCR in term of bacterial cell quantification. Real-time PCR was used to confirm the result of conventional PCR. The important thing is that four bacteria strains were detected at low concentration by simple PCR method not requiring enrichment step but only using metal hydroxides to immobilize bacteria and using DNase I to eliminate false positive result in living bacterial detection. From 10 ml samples of whole milk, bacterial cells were clarified and immobilized to be concentrated into 200 µl, resulted in 50 fold sample concentration factor. When coupled with an DNA extraction step, the sample volume was reduced 500-fold (20 µl of DNA) resulting PCR detection limits for *E. coli* O157:H7, *L. monocytogenes*, *E. coli* wild type was 5×10^1 cells and of *S. typhimurium* was 5×10^2 cells when seeded in 10 ml of whole milk by both conventional and real-time PCR. Our PCR method gave a very good sensitivity as compared to previous studies. Mostly, the bacterial detection limits did not excess 10^2-10^3 CFU/ml. Lucore *et al.* (2000) used zirconium hydroxide in bacterial immobilization to get the detection limit for *L. monocytogenes and S. enterica* of 10^2 cells/ 25 ml of whole milk with reverse transcription (RT)-PCR resulting in high copy number of molecules in viable cells. This method is very expensive compared to our method. Our result is much better than those reported by Mukhopadhyay, U.K and Mukhopadhyay, A. (2002) in that the detection limit of *E. coli* 0157:H7 and *L. monocytogenes* were found to be in the range of 10^3-10^4 CFU/ml of milk by multiplex DTD-PCR . It is also better than Nguyen *et al.* (2004) in that the detection limit is 10^3, 10^4 CFU/ml for *E. coli* O157:H7 in modified tryptic soy broth, *L. monocytogenes* in Fraser broth, respectively and 10^3 - 10^4 CFU/g of raw ground beef or hot dog, in real-time PCR.

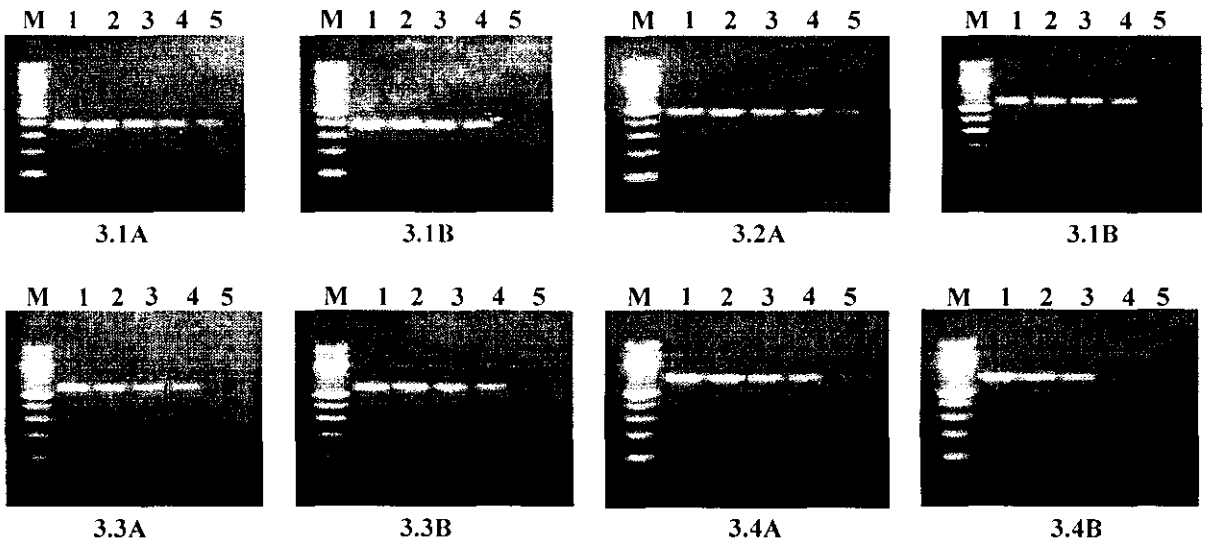

Figure 3. Detection of *E. coli* O157: H7 (3.1), *L. monocytogenes* (3.2), *E. coli* wild type (3.3) and *S. typhimurium* (3.4) from food samples (10 ml). Real-time PCR products amplified of *eaeA* (384 bp), *hly* (482 bp), *eaeA* (580 bp) and *invA* (685 bp) from cells untreated DNase I (A) and treated DNase I (B). Agarose (2%) gel electrophoresis and EtBr staining. Lane M, 100 bp DNA ladder. Lanes 1 to 5, PCR products amplified from DNA extracts corresponding to both 5×10^4, 5×10^3, 5×10^2, 5×10^1, and 5×10^0 cells of live cells and dead cells/ 10 ml whole milk, respectively.

CONCLUSION

Bacterial immobilization with metal hydroxides associated with DNase I treatment were successful to accomplish a target in bacterial detection at low concentration without requiring enrichment step with a rapid, low cost, sensitive and reliable by PCR based detection method. However, the PCR conditions can be adjusted and nucleic acid extraction protocols can be improved. The detection performance can be further improved to shorten time for DNA amplification. Also, we have illustrated that this method is applicable to detect bacteria and can be applied to a food model.

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CỐ ĐỊNH VI KHUẨN BẰNG HYDROXIDE KIM LOẠI LÀM TĂNG ĐỘ NHẠY CỦA PHƯƠNG PHÁP PCR KHI PHÁT HIỆN VI KHUẨN TRONG THỰC PHẨM

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TÓM TẮT

Vi khuẩn ở nồng độ thấp không qua nuôi dưỡng có thể phát hiện được bằng phương pháp PCR nhờ cô đặc vi khuẩn từ thực phẩm bằng hydroxide kim loại. Enzyme DNase I được sử dụng để loại bỏ DNA

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từ vi khuẩn chết và không ảnh hưởng đến DNA từ vi khuẩn sống; do đó phương pháp PCR có thể dùng để phát hiện vi khuẩn sống. SYBR green I được sử dụng trong real-time PCR để định lượng DNA được nhân lên, từ đó xác định số lượng vi khuẩn ban đầu. Tỷ lệ phần trăm vi khuẩn thu được khi cố định với hydroxide kim loại dao động từ 93,6 - 99,3% và 31,5 - 54,7% khi không cố định vi khuẩn. Trong 10 ml sữa tươi, thể tích dung dịch giảm đi 50 lần khi sử dụng hydroxide kim loại để cố định vi khuẩn từ dung dịch sữa. Kết quả làm tăng độ nhạy của phương pháp PCR để phát hiện vi khuẩn ở nồng độ thấp. Ngưỡng phát hiện của *E.coli* O157:H7 (384 bp), *L. monocytogenes* (482 bp), *E.coli* wild type (580 bp) là 5×10^1 tế bào và của *S. typhimurium* (685 bp) là 5×10^2 tế bào trong 10 ml sữa tươi bằng real-time PCR.

Từ khóa: *Cố định vi khuẩn, DNase I, real-time PCR, SYBR green I, tỷ lệ cố định vi khuẩn (%)*