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African Journal of Microbiology Research Vol. 4(23), pp. 2503-2507, 4 December, 2010
Available online http://www.academicjournals.org/ajmr
ISSN 1996-0808 ©2010 Academic Journals

Full Length Research Paper

Rapid, specific and concurrent detection of *Listeria*, *Salmonella* and *Escherichia coli* pathogens by multiplex PCR in Iranian food

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Accepted 7 October, 2010

We are unable to detect all microorganisms in media. In consequence, molecular methods like PCR based techniques can mend our difficulties in this era. Herein, we describe simultaneous detection of major food-borne pathogens *Listeria monocytogenes*, *Salmonella* spp. and *Escherichia coli* O157:H7. Iranian food materials used for comparison of traditional microbiological methods (such as culture and serology) and multiplex PCR method in the detection of pathogens were prepared from several local restaurant, including eggs, raw milk, Raw kobide, salad, chicken, and cheese. Following DNA extraction, PCR assay were performed, using three specific primer pair. Because of all different sizes of the amplified fragments for each uniplex reaction, we optimized the each primers concentration to achieve a clearly visible band pattern of agarose gel (210 bp for *Listeria*, 556 bp for *E. coli* and 942 bp for *Salmonella*). In conclusion, uniplex and multiplex PCR was considered to perceive detection of the pathogens simultaneously.

Key words: *Listeria monocytogenes*, *Salmonella* spp., *Escherichia coli* O157H7, multiplex PCR.

INTRODUCTION

Energy is not the only transferred thing from food to human. Clearly microbes especially pathogens can also be involved. World wide, Food-borne pathogens create a huge group of disease placing them in the second pace of the importance after respiratory pathogens. Totally about 16 bacterial species, 13 groups of viruses, 22 parasites and 5 kinds of single cell organisms can transmit from food to human being (Varnam et al., 2005). In food microbiology, bacteria are in greater importance in which based on CDC reports, about 70% of infections are happened by bacteria *Staphylococcus aureus*, *Salmonella* spp., *L. monocytogenes, Campylobacter jejuni* and *E. coli* in the top list (Ryan et al., 2002). *E. coli*, especially *E. coli* O157:H7 has low infection rate and can cause dysentery and hemorrhagic colitis (Hara-Kudo 2003). Isolation and detection of these bacteria from food and water is one of the challenging issues of microbiology. Nowadays, hundreds of commercial culture media are developed. Some are basal, some are differential and some others are selective. Besides, needing more time, contaminations and false positive and negatives are serious obstacles. Therefore scientists are looking for techniques to conquer these disadvantages to save time, money, and also to reach a more specificity and validity of data. In traditional detection method for *Salmonella*, we have to culture them in pre-enrichment, then enrichment and finally selective media (Vanderzant et al., 2004; Marshall et al., 2003). Sometimes complementary and confirmatory methods should be performed to observe a valid result, but even in the modern equipped laboratories this way is time consuming a key crucial issue for those patients needing quick treatments in diagnostic era (Tavakoli et al., 2008).

Fortunately, by investigating rapid, specific and accurate techniques in microorganism screening a hopeful future is expected. Molecular techniques like PCR, serological tests and biosensors technologies can help us

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Table 1. Primer pairs used in this study.

Bacteria	Target gene	Primer sequences	Reference
E. coli O157:H7	*Stx2A*	EcF: cga ggg ctt gat gtc tat cag EcR: tca gta taa cgg cca cag tcc	Keith et al., 2000
L. monocytogenes	*hly*	LmF: cgc aac aaa ctg aag caa agg LmR: ttg gcg gca cat ttg tca c	Keith et al., 2000
Salmonella sp.	*invA*	SeF: acc acg ctc ttt cgt ctg g SeR: gaa ctg act acg tag acg ctc	This work

en route for these goals.

In this paper, among the microbes *E. coli* O157:H7, *Listeria* sp. and *Salmonella* spp. were chosen based on their importance to set up multiplex PCR method for simultaneous detection of contamination in food resources.

MATERIALS AND METHODS

Bacterial strains and growth circumstance

Standard bacterial strains including *L. monocytogenes* (PTCC 1163), *Salmonella typhi* (PTCC 1609) and *E. coli* O157:H7 were prepared from microorganism collection of Veterinary Faculty of Tehran University.

Bacteria were cultured in LB broth medium (24 h in 37°C) then tested for purity, turbidity and gram staining. Afterwards, they streaked on LB agar, incubated (24 h in 37°C) and then used to test their growth and colony morphology (Scotts, 1994).

Food samples for detection

Iranian food materials used for comparison of traditional microbiological methods (such as culture and serology) and multiplex PCR method in the detection of pathogens were prepared from several local restaurant, including eggs, raw milk, Raw kobide, salad, chicken, and cheese. Each material contained two artificially contaminated samples and one naturally contaminated sample, each sample was tested with three replications, therefore in total 54 tests were performed. The overnight (18 h) grown culture of *E. coli*, *L. monocytogenes*, and *Salmonella* spp. containing approximately 90 viable cells/mL, respectively (by plate count method on corresponding selective plates) were collected by centrifugation 5 min at 3000 rpm, then 100 CFU of 1, 2, or all 3 kinds of pathogen were mixed together in 50 mL autoclaved 1% peptone solution, which was used for spiking samples homogenized to a final concentration of 100 CFU of each pathogen/mL or g, these artificially contaminated samples were taken as the positive controls to eliminate the effect of PCR inhibitors existing in food matrixes.

Genomic DNA isolation and purification

Phenol-chloroform genomic DNA extraction and boiling methods (Mac Faddin, 2000; Sambrook et al., 2001) was performed, then extraction and purification efficacy were analyzed by 1% agarose gel and by means of spectrophotometric analysis as UV absorption at 260 nm (A260) and A260/A280 ratio.

Multiplex PCR setting

In order to design primer pairs for *E. coli* and *Listeria strains*, the formerly publishing primers were chosen but for *Salmonella* spp. new primers was designed (Table 1). *Salmonella* gene *invA*, *E. coli* O157:H7 gene *stx2A* and *L. monocytogenes* gene *hly* were amplified, quantified and then the PCR products were qualified by electrophoresis agarose gel. Primer specificity test was performed on other strains as *E. coli*, *Proteus*, *Shigella* and *Enterobacter* in the same PCR condition.

All PCR reactions were performed in 30 µ total volume. Composition was prepared with primer pairs (10 pmol/µl for each primer), *Taq* DNA polymerase (10 unit/µl), dNTPs (2.5 mM of each dNTPs), PCR buffer, $MgCl_2$ (10 mM), ddH_2O and template DNA. Template concentration was optimized for each DNA sample including 2, 4, and 3 µ (concentration) for *salmonella*, *Listeria* and *E. coli*, respectively. Also PCR conditions were as follows: preincubation at 94°C for 5 mi; 30 cycles of denaturation at 94°C for 60 s, primer annealing temperature 58°C for 60 s, primer extension at 72°C for 60 s; final extension at 72°C for 3 min.

Testing food samples

Multiplex PCR process was performed based on the same condition resulted for each one and was set up based on each strains genome optimal concentration. Multiple PCR method was performed to detect these strains from artificially and naturally contaminated samples Iranian food such as milk, cheese, chicken, kabab kobide, egg and salad.

RESULTS

Application of multiplex PCR on contaminated food materials

Blast analysis of NCBI web site was used to validate the specificity and conservation of the designed primers. Besides structural analysis of primers were performed by oligo analyzer. The PCR products of *invA*, *stx2A* and *hly* genes are shown in Figure 1. The PCR product validation was also performed by sequencing of the products. No detection was observed for PCR reaction of the other strains with these designed primers. The results of multiple PCR for simultaneous amplification of three genes are shown in Figure 1.

Table 2. Traditional microbiological method vs Multiplex PCR in food samples

Food sample	Traditional method			Multiplex PCR		
	L	S	E	L	S	E
Raw-milk 1(A)	+	+	+	+	+	+
Raw-milk 2 (A)	+	+	+	+	+	+
Raw-milk 3 (N)	-	-	+	-	-	+
Egg 1 (A)	+	+	+	+	+	+
Egg 2 (A)	+	+	+	+	+	+
Egg 3 (N)	-	-	+	-	-	+
Raw kobide 1(A)	+	+	+	+	+	+
Raw kobide 2 (A)	+	+	+	+	+	+
Raw kobide 3 (N)	-	-	+	-	-	+
Chicken 1 (A)	+	+	+	+	+	+
Chicken 2 (A)	+	+	+	+	+	+
Chicken 3 (N)	-	+	+	-	+	+
Salad 1 (A)	+	+	+	+	+	+
Salad 2 (A)	+	+	+	+	+	+
Salad 3 (N)	-	-	-	-	+	-
Cheese 1 (A)	-	+	+	-	+	+
Cheese 2 (A)	-	+	+	-	+	+
Cheese 3 (N)	-	-	+	-	-	+

A = artificially contaminated samples; N = naturally contaminated samples; L = *L. monocytogenes*; S = *Salmonella* spp.; E = *E. coli* O157H7; + = positive result; – = negative result.

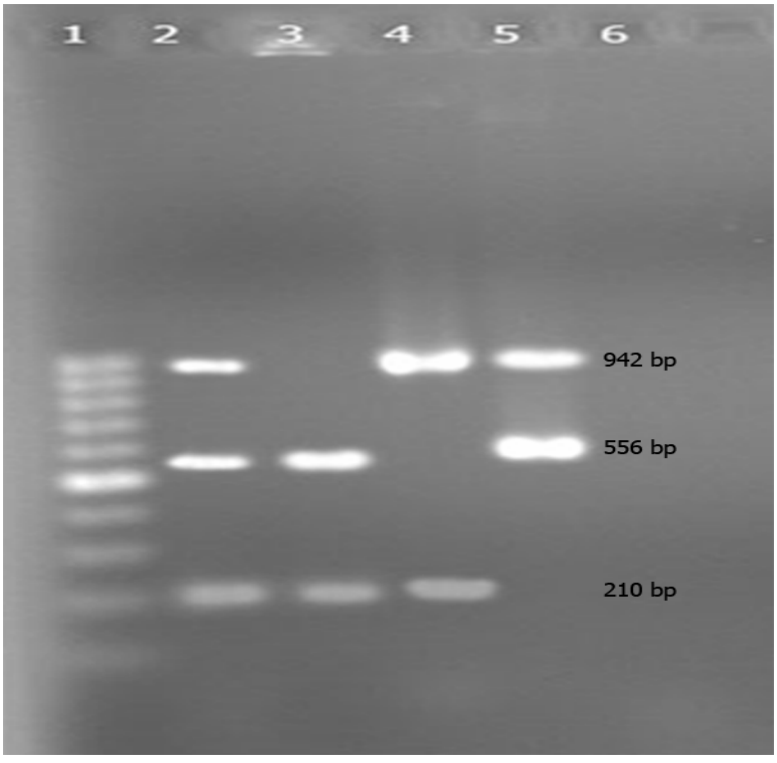

Figure 1. Simultaneous detection of three pathogenes: Lane 1: DNA ladder (100bp), Lane 2: multiplex PCR: *salmonella* (942bp), *E.coli* O157H7 (556bp), *Listeria* (210bp), Lane 3: *E. coli* O157H7 (556bp) and *Listeria* (210bp) , Lane 4: *Salmonella* (942bp), *Listeria* (210bp), Lane 5: *Salmonella* (942bp), *E. coli* O157H7 (556bp), Lane 6: negetive control.

DISCUSSION

Food microbiology covers three general areas including microorganisms' roles in food spoilage, in food production procedure and in food-borne pathogens. Nowadays considering health and medical discussions on populations, food spoilage and pathogenecity followed by food-borne disease has been under special considerations. Food poisoning is one of the new fields relating to the modern economic and health in food microbiology and food distribution. Microbiology has great influence in this era. Within, some microorganisms can produce strong toxins and therefore poisoning food. Of course all spoiled foods don't include these poisons but are probable to be involved. Unfortunately most of the poisoned foods don't show the spoilage appearance (Chuand et al., 2003; Cho et al., 2008; Beutin et al., 2009; Gaudron et al., 2009). Additionally there are many reports about population poisoning of these strains (Ryan et al., 2002; Henesy et al., 2001).

According to the pathogenic importance of these strains rapid, acute and specific detection of the strains in food would be in first priority to prevent the extension of the disease. In this reason various traditional and modern routs are included. Traditional methods are usually time-consuming and troubling for microbiologists especially when they need to give a quick detection result. For instance, detection of Salmonella in food needs several days of incubation and culturing. So physical, chemical, immunological and also molecular based methods can help us to dominate over time intensively (Calvo et al., 2008; Kawasaki et al., 2009; Keith et al., 2000; Germini et al., 2009).

Multiplex PCR is one of the rapid and trustful methods in molecular detection of pathogens. Herein we studied molecular detection of bacterial pathogens *Salmonella* spp., *L. monocytogenes* and *E. coli* O157:H7. Finally this technique was able to simultaneously detect these pathogens in food samples. We tried to set primers for simultaneous reaction of multiplex PCR. Also based on former studies, two primer pairs were selected according to the former works for *E. coli* O157:H7 and *L. monocytogenes*. We found the *Salmonella* reported primer pairs unsuitable for this work and tried to design other pairs of primers (Park et al., 2006; Omiccioli et al., 2009; Zhang et al., 2009; Kumar et al., 2009).

For multiplex PCR setting up, it's necessary to set each uniplex in optimal condition. So, we tried to set the same T_a temperature for all these primers on 58°C. Structural interferences of primers were also tested including secondary structure like dimmer and hairpins. Because of all different sizes of the amplified fragments for each uniplex reaction, we optimized the each primers concentration to achieve a clearly visible band pattern of agarose gel (210 bp for *Listeria*, 556 bp for *E. coli* and 942 bp for *Salmonella*). Also we have compared these data with other methods including differential culturing and biochemical analysis but this method is faster and more specific. Their fragment appearance in food industry in simultaneous condition can be useful (Ooka et al., 2009; Oh et al., 2009). Though several investigations have previously reported on multiplex PCR assay for detection of these pathogens in food samples. But this is the first report to optimize multiplex PCR for simultaneous detection of *Salmonella* spp., *L. monocytogenes* and *E. coli* O157:H7 in Iranian foods.

Finally we should note that besides rapid and specific detection of pathogens via multiplex PCR method, step-wise protocols should be considered to achieve a reliable data including optimized multiple primer concentrations, DNA extraction and also optimized PCR reaction condition.

ACKNOWLEDGMENT

This work was supported by the Health Research center, Baqiyatallah (a.s.) University of Medical Sciences in IRAN. Gratefully acknowledges the financial support from this research center.

REFERENCES

Beutin L, Jahn S, Fach P (2009). Evaluation of the 'GeneDisc' real-time PCR system for detection of enterohaemorrhagic *Escherichia coli* (EHEC) O26, O103, O111, O145 and O157 strains according to their virulence markers and their O- and H-antigen-associated genes. J. Appl. Microbiol., 106(4): 1122-1132.

Calvo L, Martinez-Planells A, Pardos-Bosch J, Garcia-Gil L (2008). *A* New Real-Time PCR Assay for the Specific Detection of *Salmonella* spp. Targeting the bipA Gene. Food Anal. Methods, 1(4): 236-242.

Cho K, Kambiranda D, Kim S, Math R, Lim W, Hong S, Yun H (2008). Simultaneous Detection of Food-borne Pathogenic Bacteria in Ready-to-eat Kimbab Using Multiplex PCR Method. Food Sci. Biotechnol., 17(6): 1240-1245.

Chuand M, Weyant R, Murry PR (2003). Clin. Microbiol., Ed 8 ed.

Gaudron T, Peters C, Boland E, Steinmetz A, Moris G (2009). Development of a quadruplex-real-time-PCR for screening food for genetically modified organisms. Eur. Food Res. Technol., 229(2): 295-305.

Germini A, Masola A, Carnevali P, Marchelli R (2009). Simultaneous detection of *Escherichia coli* O175:H7, *Salmonella* spp., and *Listeria monocytogenes* by multiplex PCR. Food Control., 20(8): 733-738.

Hara-Kudo J (2003). Flourogenic and chromogenic media, the rapid technique for isolation of *V. cholera*. J. Appl. Eviron. Mic., 67: 5819-5823.

Henesy T, Brod G (2001). A national outbreak of *Salmonella enteritidis* infections from ice cream. J. Med., 334: 1281- 1286.

Inatsu Y, Bari ML, Kawasaki S (2004). *Survival of Escherichia coli O157:H7, Salmonella enteritidis, Staphylococcus aureus,* and *Listeria monocytogenes* in Kimchi. J. Food Prot., 67(7): 1497-1500.

Kawasaki S, Fratamico P, Horikoshi N, Okada Y, Takeshita K, Sameshima T, Kawamoto S (2009). Evaluation of a Multiplex PCR System for Simultaneous Detection of *Salmonella* spp., *Listeria monocytogenes*, and *Escherichia coli O157:H7* in Foods and in Food Subjected to Freezing. Foodborne pathogens and disease, 6(1): 81-89.

Keith A, Palmer A, Korengay L (2000). Improved template preparation for PCR for detection of Food-borne bacterial pathogens. Appl. Environ. Microbiol., 66: 4539- 4542.

Kumar T, Murali H, Batra H (2009). Simultaneous detection of pathogenic *B. cereus, S. aureus* and *L. monocytogenes* by multiplex PCR. Indian J. Microbiol., 49(3): 283-289.

Mac Faddin JF (2000). Biochemical Tests for Identification of Medical. lippincot. 3th ed ed. Williams & Wilkins Pub.
Marshall RT (2003). Standard methods for the examination of dairy products. Sixteenth Edition ed, ed. APHA., USA.
Oh M, Paek S, Shin G, Kim H, Jung G, Oh S (2009). Simultaneous Identification of Seven Foodborne Pathogens and *Escherichia coli (*Pathogenic and Nonpathogenic) Using Capillary Electrophoresis-Based Single-Strand Conformation Polymorphism Coupled with Multiplex PCR. J. Food Protection, 72(6): 1262-1266.
Omiccioli E, Amagliani G, Brandi G, Magnani M (2009). A new platform for Real-Time PCR detection of *Salmonella* spp*., Listeria monocytogenes* and *Escherichia coli* O157 in milk. Food Microbiol., 26(6): 615-622.
Omiccioli E, Amagliani G, Brandi G, Bruce I, Magnani M (2009). Simultaneous direct detection of *Salmonella* spp., *listeRia monocytogenes and Escherichia coli* o157 in milk samples by magnetic extraction and multiplex PCR. J. rapid Meth. Automation Microbiol., 17(2): 195-213.
Ooka T, Terajima J, Kusumoto M, Iguchi A, Kurokawa K, Ogura Y, Asadulghani M, Nakayama K, Murase K, Ohnishi M, Iyoda S, Watanabe H, Hayashi T (2009). Development of a Multiplex PCR-Based Rapid Typing Method for Enterohemorrhagic *Escherichia coli* O157 Strains. J. Clin. Microbiol., 47(9): 2888-2894.
Park Y, Lee S, Kim Y (2006). Detection of *Escherichia coli O157:H7, Salmonella* spp., *Staphylococcus aureus and Listeria monocytogenes* in kimchi by multiplex polymerase chain reaction (mPCR). J. Microbiol., 44(1): 92-97.
Ryan CA, Nickels MK, Hargrett-Bean NT, Potter ME, Endo T, Mayer L (2002). Massive outbreak of antimicrobial resistant salmonellosis traced to pasteurized milk. 278, Jam. Med. ASSOS., pp. 389-395.
Sambrook J, Russell DW (2001). Molecular cloning (a laboratory manual). 4 th ed, Gold Spring.
Scotts B (1994). Diagnostic Microbiology. 9th ed ed.
Shearer AE, Strapp CM, Joerger RD (2001). Evaluation of a polymerase chain reaction-based system for detection of *Salmonella enteritidis, Escherichia coli* O157:H7, *Listeria* spp., and *Listeria monocytogenes* on fresh fruits and vegetables. J. Food Prot., 64(6): 788-795.
Tavakoli HR, Bayat M, Kosha M (2008). Application of Chromogenic media for rapid detection water and food- borne pathogens. Am. Eurasian J., 4(6): 693-699.
Vanderzant C, Splittstoesser DF (2004). Splittstoesser Compendium of methods for the microbial evaluation of foods. Third Edition ed, ed. APHA., USA
Varnam AH, Evans MG (2005). Foodborne pathogenns, an illustrated text. Manson Publications.
Zhang D, Zhang H, Yang L, Guo J, Li X, Feng Y (2009). Simultaneous detection of *Listeria monocytogenes, Staphylococcus aureus, Salmonella enterica* and *Escherichia coli* O157:H7 in food samples using multiplex PCR method. J. food Safety, 29(3): 348-363.