Archives of Razi Institute, Vol. 68, No. 2, December (2013) 105-109

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DOI: 10.7508/ari.2013.02.003

Full Article

An optimized affordable DNA-extraction method from *Salmonella enterica* Enteritidis for PCR experiments

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Received 08 Apr 2013; accepted 31 Jul 2013

ABSTRACT

In diagnostic and research bacteriology settings with budget and staff restrictions, fast and cost-effective genome extraction methods are desirable. If not inactivated properly, cellular and/or environmental DNA nucleases will degrade genomic material during the extraction stage, and therefore might give rise to incorrect results in PCR experiments. When crude cell extracts, proteinase K–treated templates and purified DNAs prepared by phenol-chloroform-isoamylalcohol method as well as a commercial extraction kit were subjected to the *Salmonella enterica* Enteritidis specific STM2 PCR, with exception of crude cell extract, PCR products from all other three methods saved their integrity for 28 days post-generation. This work aimed to find out whether improvement to boiling method can guaranty stability of PCR products. As results showed, treatment of crude cell extracts from *S.* Enteritidis with proteinase K offers an inexpensive, fast and effective DNA extraction method suitable for high-throughput laboratories.

Keywords: DNA Exrtaction, *Salmonella enterica* Enteritidis, STM2, DNase, proteinase K

INTRODUCTION

Salmonella enterica serovar Enteritidis is a major cause of Salmonellosis in both developing and developed world. In food-born infections, genotyping of pathogens is a fundamental element of any epidemiological surveillance and outbreak investigations. Preparation of high quality DNA, either genomic or plasmid, is a principal phase with significant impacts on study results. Over the last two decades a number of different strategies have been developed to provide genomic material suitable for PCR-based and non-PCR genomic analyses (Lofstrom *et al* 2011, Geng *et al* 2012, Lofstrom *et al* 2012, Quigley *et al* 2012, Vetrone *et al* 2012). Conventional DNA extraction methods are generally based on application of health-threatening and rather costly reagents such as phenolic compounds and chloroform rendering them unpleasant for laboratory workers. The very simple DNA extraction method of boiling in distilled water has found extensive

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application in molecular experiments with mycobacteria (Sweeney *et al* 2006), *Bacillus anthracis* (Dugan *et al* 2012), *Salmonella* Enteritidis (Zhang *et al* 2012), *Staphylococcus aureus* (Sowmya *et al* 2012) and many other pathogenic bacteria. A typical property of many of gram-negative bacteria of Enterobacteriaceae is carrying heat-stable deoxyribonucleases (DNase) that degrade PCR amplicons therefore, unless inactivation of DNase is considered, PCR products are not stable for a long time at 4 ºC. Treatment of DNA extracts with proteinase K and ethylendiamintetraacetate (EDTA) plus immediate storage of PCR products at -20 ºC after their amplification are known to enable inactivation of DNase activity (Gibson & McKee 1993).

In the work presented here, a cost-efficient genomic DNA extraction protocol is proposed which provides genomic DNA preparation free of PCR-inhibitors, toxic chemical and suitable for PCR-experiments from *S.* Enteritidis.

MATERIALS AND METHODS

Cultivation of bacteria and conditions of growth. Salmonella enterica serovar ATCC 13076 and an Iranian chicken isolate of *S.* Enteritidis obtained from Razi Type Culture Collection were cultured on nutrient agar plates and incubated overnight at 37°C. One loopful of each bacterial growth was transferred to 1 ml sterile TE buffer (10 mmol/L Tris-HCl (pH 8.0), 1 mmol/L EDTA) and vortexed briefly in a microfuge tube sealed with O ring.

DNA extraction. Four different methods were used as follows:

Method 1: Plain boiling. To inactivate the bacterial cells, tubes were submerged in a boiling water bath for 10 min. Inactivated bacterial suspensions were centrifuged for 5 min at 17,900 g. The supernatant was transferred to a fresh microfuge tube and stored at fridge or freezer for further use in PCR experiments.

Method 2: Modified boiling**.** DNA boilates, prepared according to the method 1, were aliquoted to four equal portions and treated with a freshly-prepared proteinase K solution (10 mg/ml) reaching the final concentration of the enzyme in tubes to 0.02 µg/ml, 0.04 µg/ml, 0.08 µg/ml and 0.16 µg/ml. Enzyme-treated dilutions were stored at fridge or freezer for further use in PCR experiments.

Method 3: Phenol-chloroform-isoamylalcohol. A loopful of each bacterial growth was transferred to an O ring-sealed microfuge tube containing 0.5 ml double-distilled water and vortexed shortly. This was followed by centrifuge for 10 min at 17,900 g .The pellet was resuspended in 700 µl of lysis buffer [50mM Tris, 50mM EDTA, 100mM NaCl, %1 SDS (pH=8)].This suspension was treated with 10 µl of proteinase K (Cinna gen Inc., Iran) (10 mg/ml) and 2 µl of RNase A (Cinna Gen Inc., Iran) and submerged for 2 hours in a water bath at 37 ºC. A volume of 700 µl of Phenol/Chloroform/Isoamylalcohol (25:24:1 ratio) was added to the enzyme-treated mixture and this was centrifuged for 10 min at 20,800 g. The aqueous portion was transferred to a fresh tube and washed twice with 500 µl of chloroform. Two volumes of isopropanol were added to the supernatant and the suspension was centrifuged for 10 min at 20,800 g. The bacterial DNA was precipitated with 2 volumes of ice-cold ethanol (70%). The precipitated DNA was then centrifuged for 10 min at 20,800 g. The DNA pellet was air-dried and redissolved in 50 µl TE buffer and stored at refrigerator or freezer until used.

Method 4: Commercial DNA extraction method. The Fermentas genomic DNA purification kit (Kit Number 0512, Fermentas, USA) was used according to the manufacturer's instructions. In brief, 400 µl of lysis buffer was added to 200 µl of overnight broth cultures of the bacteria in Triptic Soy bean Broth (TSB), and the mixture was incubated at 65 ºC for 5 min. This was followed by addition of 600 µl chloroform to the suspension that was then inverted for 3-5 times. After a round of centrifuge at 12,800 g for 2 min, the supernatant was transferred to a fresh microfuge tube and 800 µl of precipitation solution was added. The mixture was centrifuged at 12,800 g for 2 min and the supernatant was removed. One hundred microlitre of NaCl solution and 300 µl of cold ethanol (70%) were

added and the mixture was kept at -20 ºC for 10 min. A final round of centrifugation at 12,800 g for 3-4 min was conducted and the supernatant was removed and the DNA pellet was air-dried. The DNA pellet was re-dissolved in 100 µl TE buffer and kept at refrigerator (4 ºC) or freezer (-20 ºC) for further use.

STM2-PCR assay. A PCR analysis initially described by Kim et al (Kim *et al* 2006) was carried out to assess quality of extracted DNA templates and stability of PCR amplicons. This reaction targets a 171-bp long section of the genome specific to *S.* Enteritidis and is known as STM2. The 25 µl-PCR reaction mixture for each STM2 assessment comprised 12.5 µl PCR premix (2x Master Mix RED, Ampliquor, Denmark), 1 µl of each forward (5'- TCA AAA TTA CCG GGC GCA -3') and reverse (5'- TTT TAA GAC TAC ATA CGC GCA TGA A -3') primers (5 pmol/µl), 6 µl DNA template and 4.5 µl double-distilled water. The amplification protocol consisted of a single round of initial denaturation at 94 ºC for 5 min followed by 40 cycles of denaturation at 94 ºC for 30 sec, annealing at 62 ºC for 30 sec, and extension at 72 ºC for 1 min followed by a final extension phase at 72 ºC for 5 min. PCRs were performed with an Eppendorf thermocycler (Germany). PCR products were divided into three equal 8 µl-portions with the first portion analyzed immediately after amplification by 1.5% agarose gel electrophoresis with ethidium bromide staining. The remaining two portions of PCR products were stored at 4 °C for 72 hours (day 3) and 4 weeks (day 28) when similar to the first portion their integrity was analyzed by gel electrophoresis.

RESULTS

All together 7 DNA templates from each of the type strain and chicken isolate of *S.* Enteritidis was available for the PCR experiment. This included three templates from plain boiling, phenol-chloroform-isoamylalcohol extraction and commercial DNA extraction kit methods plus 4 templates from modified-boiling technique representing four concentrations of proteinase K used for enzyme treatment.

PCR findings. Based on the gel electrophoresis observations on day zero, an identical PCR product with the expected size was successfully produced by amplification of all DNA templates regardless of the extraction method used (Figure 1A). On day 3, with exception of PCR products originally from templates prepared by plain boiling, all other amplicons survived storage at 4 °C for 72 hours (Figure 1B). No visually observable difference between stability of these was detectable in gel electrophoresis. Similarly on day 28, all the PCR products amplified from DNA templates prepared by phenol-chloroform-isoamylalcohol extraction, commercial DNA extraction kit and also modified-boiling techniques proved stable in either of the type strain and the chicken isolate (Figure 1C).

DISCUSION

As a characteristic property, possession of heat-stable DNases is observed in a large number of bacterial species. *Yesinia enterocolitica* (Nakajima *et al* 1994) and *S.* Enteritidis (Gibson & Mckee 1993) are among pathogens with capability to produce DNA-degrading enzymes. In the work presented here immediate detection of products following PCR amplification of all the crude cell extracts as well as purified DNA templates confirmed successful generation of the 171-bp long target DNA fragment. Storage of PCR products at 4 ºC for 72 h however resulted in degradation of products from unpurified DNA templates. This observation indicates presence of an endogenous heat-stable DNase released from S. Enteritidis that resulted in progressive degradation of genomic bacterial DNA. We have not assessed integrity of DNA templates under study within the first 72 hours after amplification but in a similar study by Gibson and colleagues no intact PCR products was detected 48 h post PCR amplification (Gibson & Mckee 1993). In a study by Nakajima and coworkers, both pathogenic and non-pathogenic *Yersinia enterocolitica* isolates produced DNases that survived storage at 4 ºC and

were able to degrade PCR products, (Nakajima *et al* 1994) in our study we examined a laboratory strain and a pathogenic chicken isolate of *S.* Enteritidis that both displayed DNase activity. As nuclease activity has been reported in *Salmonella* Typhimurium, *Salmonella* Virchow, *Salmonella* Dublin, *Salmonella* hadar and *Salmonella* agona (Gibson & Mckee 1993), it is likely that production of thermostable DNases is a common property of all *Salmonella* spp. Comparative examination of four different concentrations of proteinase K ranging from 0.02 to 0.16 µg/ml used in modified boiling method, revealed no observably detectable difference between the integrity of corresponding PCR products. In fact identical pattern was displayed by all of these in gel-electrophoresis. A higher concentration of Proteinase K, 100 µg/ml, has been recommended elsewhere to inhibit nuclease activity in preparation of DNA templates from *Yersinia enterocolitica* (Nakajima *et al* 1994). Being a further observation of the present work, treatment of DNA templates from *S.* Enteritidis even with low concentrations of proteinase K as little as 0.02 µg/ml seemed enough to extend stability of PCR products stored at 4 ºC to at least 28 days. Using both phenol-chloroform-isoamylalcohol and commercial kit DNA extraction methods produced genomic material that were successfully amplified the expected PCR-generated product. These were stable enough to survive storage conditions for the whole 28-day examination period with no considerable loss of integrity. Despite the satisfying results provided by either of these methods, they were cumbersome, time-consuming and expensive. These drawbacks in laboratory settings with technical expertise and budget restrictions justify application of the simpler and more economic boiling methods. What the observations by present work indicate, degradation of PCR-produced material due to nuclease activity of bacterial cells might result in misleading of diagnostic and research laboratory workers in analysis of the findings. This is specifically serious when a laboratory staff is expected to decide whether a given failed PCR reaction indicates genuine absence of a specific bacterium or actually the unwelcome result of DNase activity.

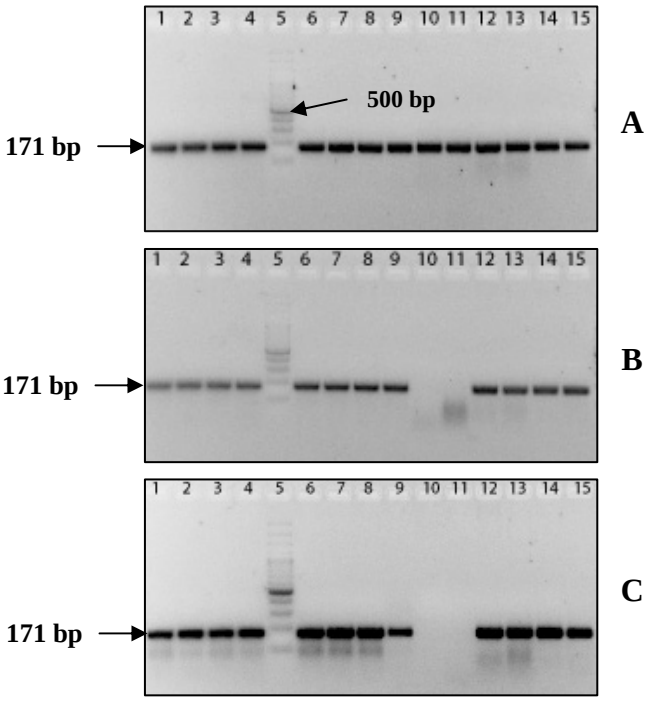

Figure 1. Gel electrophoresis analysis of PCR-generated products from amplification of the STM2 locus of *Salmonella* Enteritidis using four different DNA extraction methods (see text for details). PCR products were stored at 4 ºC during the experiment. **A.** Immediate analysis of products after PCR amplification (day 0), **B.** 72 h post-PCR amplification (day 3), **C.** Four weeks after amplification (day 28). Lanes 1-4 (*S.* Enteritidis type strain ATCC 13076) and 6-9 (*S.* Enteritidis, an Iranian chicken isolate) correspond to the modified boiling method using consecutive concentrations of proteinase K (0.02, 0.04, 0.08 and 0.16 µg/ml, respectively), Lane 5 is the 100 bp-molecular weight marker (Fermentas, GeneRuler 100 bp DNA Ladder), lanes 10 (type strain) and 11 (chicken isolate): PCR amplicons produced from plain-boiling-extracted templates, lanes 12 (type strain) and 13 (chicken isolate): PCR products generated from phenol/chloroform/isoamylalcohol extracted templates, lanes 14 (type strain) and 15 (chicken isolate): amplicons amplified from commercial kit extracted templates.

Further, in occasions when a complementary experiment such as nucleotide sequencing is required to be conducted on PCR-generated products, capacity of the DNA extraction method to enable longer storage is critical. Given the fact that these collaborating laboratories might be

geographically distant, an efficient extraction method would guaranty completion of experiments. To conclude, modified boiling would be our method of choice to prepare genomic material from *S. Enteritidis* as it is a very simple, inexpensive and fast technique for extraction that provides suitable material for PCR experiments. No specific laboratory expertise or equipment is required and the whole procedure can be accomplished within 30 min. This method is therefore, serviceable in laboratories with high throughput of samples.

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