JOURNAL OF FOOD QUALITY & HAZARDS CONTROL
SHAHID SADOUGHI UNIVERSITY OF MEDICAL SCIENCES & HEALTH SERVICES

Journal of Food Quality and Hazards Control 3 (2016) 128-133

DNA Extraction from Beef Harboring *Sarcocystis* spp.: Comparison of Three Different Analytical Methods

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HIGHLIGHTS

- Amplification of all three methods for DNA extraction of *Sarcocystis* was the same.
- The cost effective DNA extraction method for beef harboring *Sarcocystis* was salting out.
- Salting out method was introduced for DNA extraction from *Sarcocystis* spp. in beef.

Article type
Original article

Keywords
Sarcocystis
Meat
Molecular Diagnostic Techniques

Article history
Received: 12 Jul 2016
Revised: 25 Aug 2016
Accepted: 7 Sep 2016

Acronyms and abbreviations
PCI=Phenol-Chloroform-Isoamylalcohol
PAGE=Polyacrylamide Gel Electrophoresis

ABSTRACT

Background: DNA extraction is one the most important steps for molecular analysis of food-borne pathogens. In this research, three methods of DNA extractions from beef harboring *Sarcocystis* spp. were compared for the quality, quantity, safety, as well as cost-effectiveness.

Methods: About 100 mg intersostal and diaphragm were collected from 10 slaughtered cattle. After ensuring their contamination with *Sarcocyst* using Polymerase Chain Reaction (PCR) with the specific primer pair, three methods of salting out, Phenol-Chloroform-Isoamylalcohol (PCI), and commercial kit were performed. Quantification, qualification, and amplification analysis of the extracted DNA was done using spectrophotometer, agarose gel electrophoresis, and PCR, respectively. Statistical analysis was performed using ANOVA test, by SPSS, Inc, Chicago, IL software (v.16.0).

Results: Qualification in all methods was appropriate but the ones related to salting out and PCI methods were the best in comparison with the ones from commercial kit. Quantification analysis indicated the mean concentration of 249.3±3.94, 67.8±5.1, and 31±2.7 ng/µl for PCI, salting out, and commercial kit, respectively. The purification analysis represented the mean ratios of A (260)/(280), 1.7±0.3, 1.63±0.2, and 1.81±0.6 for PCI, salting out, and commercial kit, respectively. No significant difference (p>0.05) was found between the yielded concentration and purification among three methods.

Conclusion: The commercial kit is expensive, but salting out and PCI methods are cost effectiveness, however the last is considered as a toxic method. Because, amplification in all methods was appropriate, we introduced salting out for molecular detection of *Sarcocystis* in beef.

Introduction

Food-borne diseases have been increased over the last decades especially in developing countries and therefore have been known as a major public health problem worldwide (Oliver et al., 2005; Van de Venter, 2000). The common infection with food-borne diseases is the consumption of contaminated food or water with pathogens including, bacteria, viruses, fungi, and parasites (Zhao et al., 2014). One of the important ways which

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To cite: Eslami G., Manafi L., Peletto S. (2016). DNA extraction from beef harboring *Sarcocystis* spp.: comparison of three different analytical methods. *Journal of Food Quality and Hazards Control*. 3: 128-133.

infects human beings by food-borne diseases is consumption of raw or undercooked foods such as sea-foods, meat, and poultry (Omurtag et al., 2013; Rosec et al., 2012; Wingstrand et al., 2006). It is obvious that in order to prevent food-borne diseases, food analysis for the presence of pathogens is essential. The common methods for food analysis, especially meat, are usually inexpensive and simple but have some limitation in detection and species identification (Lee et al., 2014). Low sensitivity and specificity could increase the chance of food-borne pathogens. There are many methods with high sensitivity and specificity for detection as well as identification of food-borne pathogens transmitted by meat. The most important techniques is molecular PCR based methods (Boughattas and Salehi, 2014; Zhao et al., 2014). DNA extraction as the primary step for molecular analysis of food-borne pathogens is crucial. There are various commercial kits for DNA extraction from meat as a biological sample for detection and identification of food-borne pathogens. But, application of these methods in developing countries especially when large size samples are analyzed is not affordable.

Sarcocystis spp. with a high prevalence in cattle is a two-host obligatory parasite, belonging to Apicomplexa (Ghisleni et al., 2006). Cattle are its intermediate host and therefore the cysts are formed in muscle, diaphragm, etc. Therefore, in order to control and prevent the meat-borne diseases, meat inspection has an important role. The *Sarcocystis* spp. which exists in beef has various species but some of them could be pathogenic for human beings (Fayer et al., 2015). Identification of pathogenic species in meat inspection is not possible with routine tests such as microscopic analysis. Actually, the only approach for species identification is application of PCR-based molecular tools. Therefore, for achieving the best results, the extracted amplifiable DNA with appropriate quality and quantity is necessity.

The main purpose of this study was to introduce an appropriate method with low cost, fast, high quality, and amplifiable extracted DNA from beef to conduct molecular analysis. So, we compared quality, quantity, and amplifiability of three methods of commercial kit, salting out, and Phenol-Chloroform-Isoamylalcohol (PCI) for DNA extraction from beef contaminated with *Sarcocyst* spp.

Materials and methods

Sample

About 100 mg intercostal and diaphragm samples were obtained from 10 slaughtered cattle in slaughterhouse, Yazd and immediately transferred to the laboratory.

Molecular detection of Sarcocystis spp.

DNA extraction with high quality of commercial kit (Qiagen, USA, # 69504) was performed from 30 mg of each sample. The quality and quantity of extracted DNA were measured using agarose gel electrophoresis (Akhtariyan, Iran) as well as spectrophotometer (Biophotometer, Eppendorf), respectively.

For selection of the beef harboring the protozoan *Sarcocystis* spp., molecular detection was carried out using the specific primer pair of SAR-F: 5'-TGGCTAATACATGCGCAAATA-3' and SAR-R: 5'-AACTTGAATGATCTATCGCCA-3' by conventional PCR. The amplicon with the size of about 170 bp was considered as *Sarcocystis* spp. The post-PCR analysis was also performed using Polyacrylamide Gel Electrophoresis (PAGE) with ethidium bromide staining. All samples harboring *Sarcocystis* spp. were floated in 70% alcohol and stored at -20 °C for next steps.

DNA extraction with PCI method

The modified PCI method was performed as recommended by Sambrook and Russel (2006a). Briefly, 30 mg of the beef contaminated with mentioned protozoa was applied for lysis step. The lysis buffer (500 µl for each sample) was NET (NaCl, 50 mM; EDTA pH 8, 25 mM; Tris-HCl pH 7.6, 50 mM) buffer (Hajimohammadi et al., 2014). RNase treatment was carried out using the mentioned enzyme with a final concentration of 100 mg/ml and incubated for 30 min at 37 °C (Eslami et al., 2011). For the best lysis of the beef's proteins, 20 µl of proteinase K (10 mg/ml solution) was added to the solution for a final concentration of 400 µg/ml. The solution was then incubated at 56 °C overnight on the rotated stirrer. For purification, an amount of 500 µl equilibrated-Phenol:Chloroform:Isoamylalcohol (25:24:1) was added to each microtube and mixed gently for 5 min. Centrifugation was performed at room temperature at high speed (16600 xg) for 5 min. The aqua's phase of each sample was carefully transferred to a new sterile 1.5 ml microtube. The solution of equilibrated-Phenol: Chloroform (25:25) in a volume of 500 µl was added to each sample. Each sample was then mixed and centrifuged. Again, the aqua's phase was transferred to another new sterile 1.5 ml microtube. The volume of 500 µl chloroform was added to each sample. Centrifugation was repeated similar to the above condition for 1 min. The aqua's phase containing nucleic acid was transferred to a new sterile 1.5 ml microtube. The precipitated step was conducted using 1 ml cold absolute ethanol alongside with 50 µl of 3 M sodium acetate, pH 6.0, and incubated on ice for 30 min. Subsequently, centrifugation was carried out at 16600 xg for 10 min at 4 °C. The supernatant

containing alcohol was removed and the DNA pellet was washed with 250 µl cold ethanol (70%). After mixing gently, each sample was centrifuged for 10 min at 16600 xg. The supernatant was discarded and then incubated at room temperature for about 5 min till the sample damped, the appropriate amount of double distilled sterile water (100 µl) was added and incubated at 56 °C for 1 h. The extracted DNA was stored at -20 °C for further analysis.

DNA extraction with salting out method

The salting out method was performed based on the method recommended by Sambrook and Russel (2006b) with modification. Briefly, 30 mg of the beef with the mentioned parasite was used for lysis step. The lysis buffer (500 µl for each sample) was NET (NaCl, 50 mM; EDTA pH 8, 25 mM; Tris-HCl pH 7.6, 50 mM) buffer (Hajimohammadi et al., 2014). RNase treatment was performed using the mentioned enzyme with a final concentration of 100 mg/ml and incubated for 30 min at 37 °C. For the best lysis of the proteins of beef, 20 µl of proteinase K (10 mg/ml solution) was added to the solution for a final concentration of 400 µg/ml. The solution was then incubated at 56 °C an overnight on the rotated stirrer. For purification, an amount of 300 µl saturated NaCl (6 M) was added to each microtube and mixed gently for 5 min. Centrifugation was carried out at room temperature at high speed (8000 xg) for 5 min. The aqua's phase of each sample was carefully transferred to a new sterile 1.5 ml microtube. The precipitated step was conducted using 1 ml cold absolute ethanol and then incubated on ice for 30 min. Subsequently, centrifugation was performed at 16600 xg for 10 min at 4 °C. The supernatant containing alcohol was removed and the DNA pellet was washed with 250 µl cold ethanol (70%). After mixing gently, each sample was centrifuged for 10 min at 16600 xg. The supernatant was discarded, then the sample was incubated at room temperature for about 5 min till it was damped, after that an appropriate amount of double distilled sterile water (100 µl) was added and incubated at 56 °C for 1 h. The extracted DNA was stored at -20 °C for next investigations.

DNA extraction with kit

The commercial kit used in this step was the DNA extraction kit of Bioneer (Korea, # KB-3032) that was performed based on manufacturer's instruction.

Analysis of extracted DNA

The qualification analysis was determined using spectrophotometer at wavelength of 260 and 280 nm. The purification of the extracted DNA was conducted by OD260/OD280 ratio.

The quantification analysis of the extracted DNA was performed using 0.7% agarose gel electrophoresis. The results were visualized using gel documentation (E-Gel® Imager, life technologies).

The genomic DNA extracted from all methods was amplified using the specific primer pair of SAR-F: 5'-TGGCTAATACATGCGCAAATA-3' and SAR-R: 5'-AACTTGAATGATCTATCGCCA-3' for detection of *Sarcocystis* spp. (Vangee et al., 2007). The amplicons were analyzed using PAGE with ethidium bromide staining.

Statistical analysis

Each method was carried out in triplicate. Statistical analysis was performed using ANOVA test, by SPSS, Inc, Chicago, IL software (v.16.0). Significant differences were considered statistically at the 95% confidence level ($p<0.05$). All analysis were done triplicate and the data measured as mean±Standard Deviation (SD).

Results

Qualification analysis using agarose gel electrophoresis (0.7%) showed that all methods could extract genomic DNA without any fragmentation and therefore were suitable for molecular techniques, but the ones extracted from PCI method were sharpest (Fig. 1).

Quantification analysis using spectrophotometer showed that all methods had appropriate yields (Table 1). The DNA extraction analysis at wavelength of 260 nm showed the mean concentration of 249.3±3.94, 67.8±5.1, and 31±2.7 ng/µl for PCI, salting out, and commercial kit, respectively. The purification analysis using the mean ratio of a 260/280 from the extracted DNA was 1.7±0.3, 1.63±0.2, and 1.81±0.6 for PCI, salting out, and commercial kit methods, respectively (Table 1). The statistical analysis for quantification analysis showed no significant differences ($p>0.05$). The amplifiable analysis was performed using conventional PCR, consequence of PAGE, and ethidium bromide staining. Data represented that all extracted DNA were amplifiable, resulted in sharp and specific fragment with length of about 170 bp (Fig. 2).

Table 1: Efficiency of the studied DNA extraction methods with quantification analysis using spectrophotometer. The extracted DNA concentration was measured using OD 260 and purification was done using the ration of OD260/OD280

Methods	DNA concentration (ng/µl)	Purification OD260/OD280
PCI	249.3±3.94	1.7±0.3
Salting out	67.8±5.1	1.63±0.2
Commercial kit	31.0±2.7	1.81±0.6

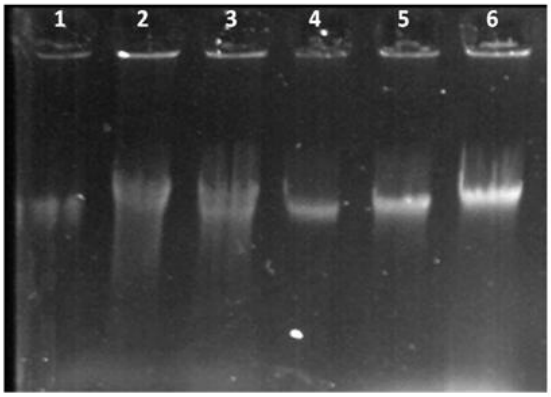

Fig. 1: Agarose gel electrophoresis (0.7%) of the genomic DNA for qualification analysis. Lanes 1 and 2: extracted DNA using commercial kit (Bioneer); lanes 3 and 4: extracted DNA using salting out method; lanes 5 and 6: extracted DNA using PCI method

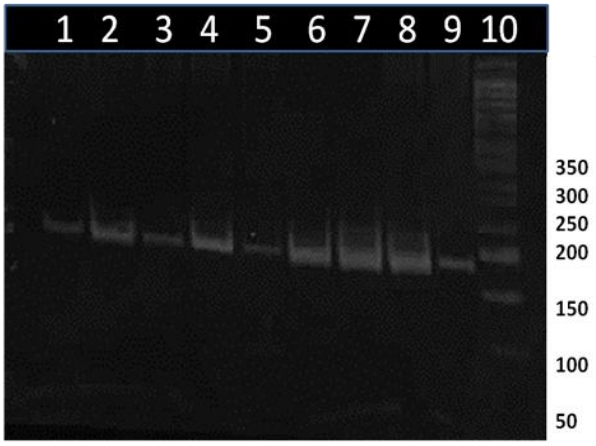

Fig. 2: PAGE analysis (15%) with ethidium bromide staining. Lanes 1-3: the amplicons resulted from salting out; lanes 4-6: the amplicons resulted from extracted DNA using commercial kit (Bioneer); lanes 7-9: the amplicons resulted from salting out method; lane 10: 50 bp DNA ladder. Amplicon size was about 170 bp

Discussion

In this study, three methods were used for DNA extraction from beef infected with *Sarcocystis* including, PCI, salting out, and commercial kit. Results showed that all extracted DNA from different methods had some variations in quantity and quality but statistical analysis showed no significant differences. The amplification analysis using conventional PCR showed suitable amplification. This data showed there was not any inhibitor in the extracted DNA from all the studied methods. The findings obtained from the current study showed that the yield of these three methods is the same that confirmed the results of the study by Mirmomeni et al. (2010). They used three methods of PCI, commercial kit, and salting out using ammonium acetate for DNA extraction from paraffin-embedded tissue. They also showed that all three methods have the same yield for DNA extraction from this kind of sample.

The commercial kits are usually the expensive ones, but PCI and salting out methods were considered with low cost. In comparison with PCI and salting out, our study showed that salting out could be introduced as the best one because of low toxicity and appropriate efficiency that confirmed the study done by Maurya et al. (2013). They compared three methods of DNA extraction including, QIAamp DNA Mini kit, PCI, and salting out from whole blood that their results showed the salting out method is simple, fast, and cost efficient. Also, Javadi et al. (2014) also reported that salting out is a non-toxic and time-effectiveness method in comparison with PCI and it is recommended to use this method in laboratories. The commercial kits have some especial buffers for the best results in lysis and purification but they are very expensive. Therefore, despite of their power for yielding pure extracted genomic DNA, their application in parts of the world with low incomes and developing countries especially whenever a large size of samples should be analyzed is not recommended. These lysis buffers mostly contain chaotropic salt that with disruption of hydrogen bonding network can destroy the stability of macromolecules such as proteins and nucleic acids (Bhaganna et al., 2010). One of the most important chaotropic salts is guanidinium chloride that is used in many genomic DNA extraction commercial kits (Eaglestone et al., 2000). It is very toxic and mutagenesis and therefore during the work, consumers need to be very careful. The chaotropic agents are not applied normally in common lysis buffer used in PCI and salting out methods (Kim and Morrison, 2009). The lysis buffer used in this study for PCI and salting out contained NaCl, 50 mM; EDTA pH 8, 25 mM; Tris-HCl pH 7.6, 50 mM that despite of very dangerous chaotropic agents, are safe.

The purification step are consisted of special column with silica powder for the commercial kit, Phenol-Chloroform for PCI method, and saturated salt (NaCl) for salting out method. Purification method is a crucial step since it removes any other molecules besides nucleotides. As shown in Table 1, the purification analysis showed the mean ratios (260)/(280) of 1.7±0.3, 1.63±0.2, and 1.81±0.6 for PCI, salting out, and commercial kit, respectively. Therefore, it seems that the purification of commercial kit is the best followed by PCI and salting out methods. In commercial kit method, chaotrope agents with destroying of the association between nucleic acid and water resulted in binding DNA to the silica powder in the column (Boom et al., 1990). On the other hand, facilitation of protein solubilization is caused by the detergents presented in lysis buffer. Therefore, during the washing step, DNA molecules bind to the column and others such as proteins, salts, as well as polysaccharides leave the silica layer. Purification in

salting out method could recover the genomic DNA with the least purity in comparison with the PCI and commercial kit. This result confirms the outcomes achieved by Freschi et al. (2005), Asadzadeh et al. (2010), and Davoudi et al. (2012). However, all extracted DNA had the appropriate purification suitable for PCR. In other words, all inhibitory agents such as phenolic materials, alcohol, etc were removed during the purification process (Sambrook and Russell, 2006a; Tan and Yiap, 2009). On the other hand, because one of the important targets of this study was selection of the safe method, therefore it seems that salting out could be a selective one for DNA extraction since it has the safe material during the lysis and purification steps.

The present study showed that DNA concentrations were different among three methods. PCI showed the highest yield of DNA and the commercial kit represented the lowest one. It seems that commercial kit made more purification than the other studied methods but the yield of DNA was the least. DNA extracted using PCI method was in high concentration but since DNA extraction in this method was considered time consuming and also due to its toxic material used in this method, it is not recommended for DNA extraction from meat harboring *Sarcocystis* spp. especially in large sample size analysis. Salting out had medium concentration and purification with appropriate amplification using specific primer pair for *Sarcocystis* spp., therefore, it is recommended for molecular PCR based method for analysis the meat contaminated with *Sarcocystis* spp.

Conclusion

Our study showed that all the three studied methods are useful for DNA extraction from meat contaminated with *Sarcocystis* spp., but salting out method is recommended due to its better safety, and affordability, as well as less time consuming in comparison with PCI method.

Conflicts of interest

There is no conflict of interest.

Acknowledgements

This study was extracted from Msc student thesis and funded by Faculty of Health, Shahid Sadoughi University of Medical Sciences, Yazd, Iran.

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