

Full Length Research Paper

A rapid and low-cost DNA extraction method for isolating *Escherichia coli* DNA from animal stools

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The price of commercial DNA extraction methods makes the routine use of polymerase chain reaction amplification (PCR) based methods rather costly for scientists in developing countries. A guanidium thiocyanate-based DNA extraction method was investigated in this study for the isolation of *Escherichia coli* (*E. coli*) DNA from goat, chicken, pig, cow and human stool samples. Two versions of the lysis buffer, with and without α -casein, were tested to alleviate PCR inhibition associated with DNA isolated from stool samples. Results obtained show that, this method using the lysis buffer containing α -casein, produces PCR ready DNA at a fraction of the cost of commercial DNA extraction kits.

Key words: DNA extraction, *Escherichia coli*, polymerase chain reaction amplification (PCR), stool samples.

INTRODUCTION

Traditional culture based techniques for the direct isolation and identification of bacterial food or waterborne pathogens from stool specimens are time-consuming and laborious (Fukushima et al., 2003; Nechvatal et al., 2008). This increases if the stool sample needs to be screened for various organisms. Molecular biological techniques, such as the polymerase chain reaction (PCR), are increasingly being used to detect and study the occurrence of microbiological agents in both human and animal stool (Silkie and Nelson, 2009; Tang et al., 2008) although, most studies are directed toward the study of pathogenic bacteria in human stool samples (McOrist et al., 2002; Shelton et al., 2008). The research approach taken, range from extraction of bacterial DNA from stool samples (Nechvatal et al., 2008; Trochimchuk et al., 2003), with and without prior enrichment, followed

by PCR (Silkie and Nelson, 2009; Wilke and Robertson, 2009), real-time PCR (Caldwell and Levine, 2009; Chasange et al., 2009) as well as density gradient gel electrophoresis (DGGE) to study the isolated DNA (Sharma et al., 2003; Zoetendal et al., 2001).

The successful application of techniques such as polymerase chain reaction (PCR) and reverse transcriptase-PCR depends largely on the integrity, yield and purity of the isolated DNA (Chasange et al., 2009; Silkie and Nelson, 2009; Yu and Morrison, 2004). Factors such as the incomplete lyses of the bacterial cells and the presence of PCR inhibitors in the samples are two of the major concerns when choosing the proper DNA extraction method (Malorny and Hoorfar, 2005). Various studies have been performed to overcome these hurdles and include the removal of PCR inhibitory substances by improved DNA purification protocols or by the addition of PCR facilitators such as bovine serum albumin (BSA) (Al-Saud and Radstrom, 2000).

Published methods employed for the isolation of bacterial DNA from stool samples are usually directed towards only one source of faecal samples and utilise commercial kits such as the QIAamp[®] DNA stool purification kit (Fukushima et al., 2003; Gioffré et al.,

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Abbreviations: PCR, Polymerase chain reaction; RT-PCR, reverse transcriptase-PCR; BSA, bovine serum albumin.

2004; Holland et al., 2000). Other authors reported on the use of various combinations of physical, chemical and commercially available bacterial cell disruption methods for the release of bacterial DNA from bacterial cells in the faecal matrix (Sharma et al., 2003; Yu and Morrison, 2004; Zoetendal et al., 2001).

Mieta et al. (2010) reported on the use of the a modification of the guanidium thiocyanate method reported by Boom et al. (1990), combined with homemade spin columns reported by Borodina et al. (2003) for the isolation of bacterial DNA from human stool samples. The author reported that, this method was successful in isolating PCR amplifiable DNA from pathogenic *Escherichia coli*, *Salmonella*, *Vibrio* and *Shigella* species directly from human stool samples at a fraction of the cost of commercial kits.

This method, with and without the addition of α -casien to the lysis buffer (Boom et al., 1999), was tested for its ability to isolate PCR-ready bacterial DNA from various animal stools (human, pig, chicken, goat and cattle) using *E. coli* as representative for bacterial pathogens tested by Mieta et al. (2010).

MATERIALS AND METHODS

Bacterial strains

An environmental isolate confirmed by API 20E (Omnimed) and PCR as commensal *E. coli* was used for all experiments, when *E. coli* was added to the samples (spiking). The bacterium was sub-cultured on plate count agar plates (Oxoid) at 37°C for 16 h. Liquid cultures were grown in Nutrient broth (Oxoid) at 37°C for 16 h with mild agitation.

Sampling of stools

Faecal samples were collected from four species of wild animal namely chicken, goat, cattle and pigs in rural communities of the Vhembe region in the Limpopo Province, South Africa. Human stool samples that were tested were received as part of a bigger study by the University of Venda with ethical clearance. Fresh faecal matter from humans and animal sources were directly and aseptically transferred into a storage container and kept at 4°C until used. A total of 20 stool samples were collected which represented four samples of each of the five faecal sources.

Stool specimen preparations for DNA extraction

An amount of 0.15 to 0.18 g of the faecal matter was aseptically placed into a 2 ml microtube (Whitehead scientific) containing 1.5 ml of sterile distilled water (Fukushima et al., 2003). The sample was then suspended in the water, mixed using a vortex and incubated at 32°C for 30 min to assist with the resuspension of the stool. After incubation of the sample, re-suspended was performed again by vortex and centrifuged for 15 s at 13000 x g to remove the debris. The supernatant was transferred into a clean microtube and either frozen or used immediately for the DNA purification step.

DNA extractions from stool samples

The method reported by Mieta et al. (2010) was used for this section with the following modifications. DNA extraction was performed using 200 μ l stool sample suspension as template. The lysis buffer was pre-mixed with the celite and the lysis step was performed at 70°C. The DNA was eluted with 400 μ l elution buffer (Qiagen).

Two versions of the lysis buffers were used. The first was the original lysis buffer (5.25 M GuSCN, 50 mM Tris-HCl, pH 6.4, 20 mM EDTA, 1.3% (wt/vol) Triton X-100) reported by (Mieta et al., 2010) termed L6 as well as the lysis buffer L7a containing 1 mg/ml α -casein (catalog no. C6780; Sigma-Aldrich Chemie, South Africa) (Boom et al., 1999). All buffers were prepared and cleaned as reported by Boom et al. (1999 and 1990).

Evaluation of DNA extraction methods

Evaluation of DNA recovery

This experiment was performed to evaluate the DNA recovery during the DNA extraction method in the presence of the faecal matter. The recovery of DNA during the DNA extraction in the presence of the faecal matter was assessed by spiking the animal and human stool samples with known quantities of extracted *E. coli* DNA (~70 ng) before the DNA extractions were performed. A total of 20 stool samples were used which represented four samples of each of the five faecal sources. The extracted DNA was tested for the presence of the *E. coli* DNA (used as spiking agents) as well as the presence of PCR inhibitors with the *E. coli* specific PCR. The extracted DNA was subjected to DNA quantification and PCR to estimate the DNA recovery and PCR inhibition for each system.

Evaluation of DNA extraction efficiency

This experiment was performed to evaluate the efficiency of the DNA extraction methods for the lysis of the bacterial cells in the presence of faecal matter. Animal and human stool samples were spiked with 10 μ l of an overnight *E. coli* culture and the protocols were followed as described earlier. The extracted DNA was subjected to DNA quantification and PCR to estimate the DNA recovery and PCR inhibition for each system.

Testing of DNA extraction methods on stool samples

DNA was extracted from unspiked stool samples and the PCR was performed to evaluate the protocols in terms of native *E. coli* DNA in the samples.

Escherichia coli specific polymerase chain reaction

The extracted DNA from the animal stool samples was examined for the presence of *E. coli* using the polymerase chain reaction (PCR) with specific primers designed for the *E. coli* malate dehydrogenase (*Mdh*) gene reported by Noller et al. (2003) to be present exclusively in all *E. coli*. PCR reactions were performed in a Biorad Mycycler™ Thermal cycler in a total volume of 20 μ l. Each reaction consisted of 10 x PCR buffer; 2 μ l extracted DNA; 2.5 mM dNTP's; 2.5 pmol each of the *Mdh* forward primer (5'-GGT ATG GAT CGT TCC GAC CT-3') and *Mdh* reverse primer (5'-GGC AGA

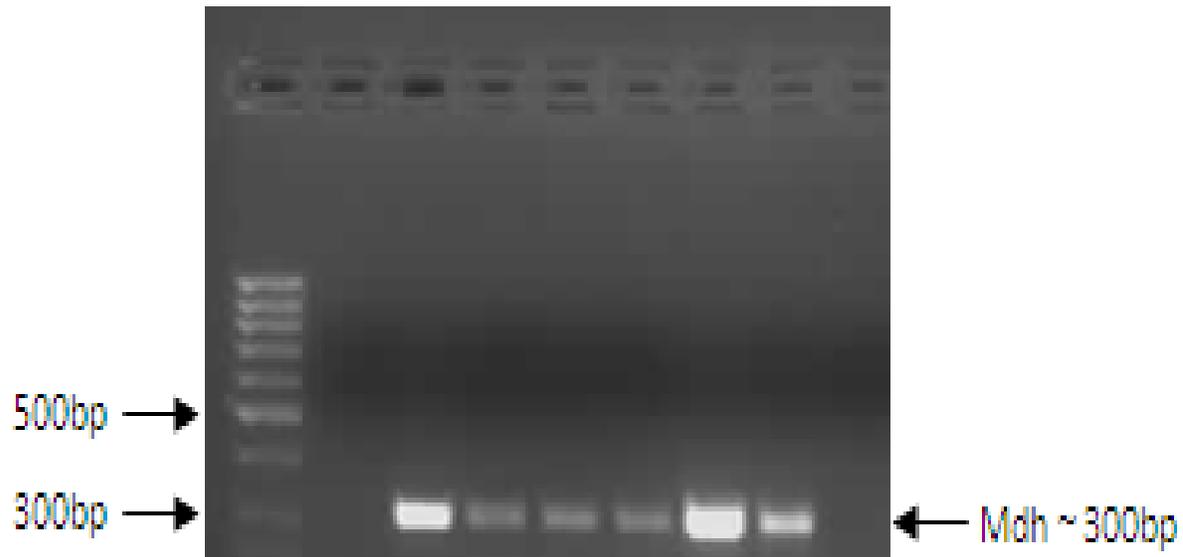


Figure 1. 1% Agarose gel showing typical PCR results obtained for the amplification of the *Mdh* gene after the DNA extraction experiments. The 100 bp molecular weight marker is loaded in lane 1, the negative control in lane 2 with the positive control in lane 3 and lanes 4 to 8 PCR results for some of the samples tested.

ATG GTA ACA CCA GAG T-3'); 0.5 U Hotstar Taq polymerase (Qiagen®) and PCR grade water. Bovine serum albumin (BSA) was added to the PCRs for DNA extracted with Buffer L6 to lift possible PCR inhibition introduced by the stool samples.

The reactions were subjected to one cycle at 95°C for 15 min, followed by 35 cycles consisting of 94°C for 45 s, 55°C for 45 s and 72°C for 1 min with a final elongation at 72°C for 5 min.

DNA electrophoresis

DNA was analyzed on a horizontal agarose slab gel (1 % (w/v)) with ethidium bromide (0.5 µg/ml) in TAE buffer (40 mM Tris acetate; 2 mM EDTA, pH 8.3). Electrophoresis was done for 1 to 2 h in electric field strength of 80 V/cm gel and the DNA was visualized with UV light (Gene Genius Bio Imaging system, Vacutec®). The O'GeneRuler™ 100 bp DNA ladder (Fermentas) was run on each gel to estimate the size of the PCR products.

DNA concentration determination

The DNA concentration of the extracted DNA was determined using the Qubit™ fluorometer (Invitrogen™) with the Quanti-iT™ ds DNA BR assay kit (Invitrogen™). All assays were performed according to the manufacturers' instructions using 20 µl extracted DNA for each reaction.

Data analysis

All experiments were performed in triplicate, with negative template controls (where water was substituted for *E. coli* DNA) and positive template controls (where samples were spiked with confirmed commensal *E. coli* DNA). Overall, the average mean of DNA yield was reported.

RESULTS AND DISCUSSION

PCR analysis of samples

DNA extracted from all the samples were tested for the effect of PCR inhibitors using a PCR targeting the *E. coli Mdh* gene (Figure 1). DNA extracted with Buffer L6 and L7a did not show PCR inhibition except for two samples each in the first two experiments. Adding BSA to the DNA samples extracted with Buffer L6 and L7a contributed to the PCR inhibition in some of the samples (data not shown).

Evaluation of DNA recovery

Results are reported in Table 1. No conclusions about the recovery rate could be determined since DNA from other bacteria present in the samples could also be isolated influencing the DNA yields. This was indeed the case with DNA isolated using the Lysis buffer L6 and L7a where the majority of the samples showed DNA concentrations higher than 70 ng.

The *Mdh* gene could be successfully amplified from 95% (19/20) of the DNA isolated using lysis buffer L6. Results obtained for lysis buffer L7a showed the successful amplification of the *Mdh* gene from 90% (18/20) of the samples tested. The samples that gave the most problems were that of the goat stool samples showing varying degrees of negative results in both the DNA extraction methods.

Table 1. Results obtained for the DNA recovery experiments showing the average DNA yields obtained, as well as number of samples for which the *Mdh* gene could be amplified. Stool samples were spiked with ~70 ng extracted *E. coli* DNA before the DNA extractions were performed.

Source	n*	Lysis buffer L6		Lysis buffer L7a	
		PCR ¹	DNA yield (ng) ²	PCR ¹	DNA yield (ng) ²
Faecal samples spiked with extracted <i>E. coli</i> DNA					
Pig	4	4	209.2 (100.4 ; 322)	4	245.2 (76 ; 532)
Chicken	4	4	80.3 (60.8 ; 100.4)	3	160.4 (58.8 ; 294.6)
Goat	4	3	577.9 (267.6 ; 832)	3	472.2 (316.8 ; 600)
Cattle	4	4	77 (69.6 ; 92)	4	150.2 (76.8 ; 322.8)
Human	4	4	121 (76.8 ; 220.4)	4	92.2 (72.9 ; 106.4)
Faecal samples spiked with <i>E. coli</i> cells					
Pig	4	4	92 (72 ; 136.8)	4	128.7 (88 ; 183.6)
Chicken	4	4	130.6 (103.2 ; 177.6)	4	148 (101.2 ; 189.2)
Goat	4	3	92.7 (0 ; 186.8)	4	114.2 (0 ; 170.4)
Cattle	4	4	98.1 (74.4 ; 115.6)	4	108.5 (46.4 ; 197.2)
Human	4	4	93.8 (76.8 ; 112.4)	4	121.4 (80.4 ; 176)
Faecal samples not spiked with <i>E. coli</i>					
Pig	8	1	Not determined	3	Not determined
Chicken	8	2	Not determined	2	Not determined
Goat	8	1	Not determined	5	Not determined
Cattle	8	0	Not determined	6	Not determined
Human	8	1	Not determined	4	Not determined

*Number of stool samples tested; ¹number of samples testing positive for the *Mdh* gene; ²average mean (min; max).

Evaluation of DNA extraction efficiency

Animal and human stool samples were spiked with 10 µl culture broth from a culture of *E. coli* grown overnight, before the DNA extractions were performed to investigate the possible effect, the faecal matter might have on the DNA extraction method. The number of cells in the aliquot of cultured broth was determined to result in approximately 70 to 90 ng DNA as determined with the direct DNA isolation from the cells using lysis buffer L6.

A total of 20 stool samples were used which represented four samples of each of the five faecal sources. The extracted DNA was tested for the presence of the *E. coli* DNA used as spiking agent as well as the presence of PCR inhibitors with the *E. coli* specific PCR and the results can be seen in Table 1. Similar to the situation in the previous section the DNA concentrations were determined to evaluate the efficiency of the various methods.

Lysis buffer L7a had a 100% (20/20) DNA extraction efficiency in comparison to lysis buffer L6 (95%; 19/20). The DNA concentrations obtained from the isolated DNA with lysis buffer L6 and L7a was comparable for all the samples tested. Once again, there was one goat sample for which no DNA concentration could be determined for both the lysis buffer L6 and L7a methods but that still gave a positive PCR result for the samples isolated with

lysis buffer L7a.

Testing of DNA extraction methods on stool samples

DNA was extracted from 40 stool samples using the DNA extraction methods without the addition of any *E. coli* cells or DNA for a comparison of the extraction efficiencies of the two methods in terms of the native *E. coli* present in the sample. The commencing 40 samples represented 8 samples from each faecal source and were tested for the presence of *E. coli* in the samples using the *E. coli* specific PCR. The results obtained for this experiment can be seen in Table 1. The results obtained showed that, for the unspiked samples the *E. coli Mdh* gene could be amplified from 50% (20/40) of the sample DNA isolated using lysis buffer L7a and 12.5% (5/40) using lysis buffer L6.

Conclusion

The purpose of this study was to test the method reported by Mieta et al. (2010) for the isolation of bacterial DNA from faecal material from both humans and animals. The decision was taken to compare the use of the

addition of α -casein to the lysis buffer (Boom et al. 1999) with the lysis buffer reported by Mieta et al (2010). Chicken, goat, pig, cattle and human stool samples were collected in the Vhembe region of the Limpopo Province.

The two methods gave comparable results when either *E. coli* DNA or cells were added to the samples prior to the DNA extraction procedure. In the case of the unspiked samples, it was clear that the use of lysis buffer L7a were a better choice for the isolation of DNA from animal stool samples. Similar to reports by Tang et al. (2008) it was noted that, stool storage conditions play an important role on the detection of bacterial pathogens in stool samples. This was especially the case for chicken and goat faecal matter that is excreted in a semi-dry form, leading to problems with getting these stool samples dissolved in the buffer due to the dry nature of the stool. This alone had a drastic influence on the extraction of bacterial DNA associated with the faecal matter (Silkie and Nelson, 2009). It should thus, be emphasized that all DNA extraction methods using animal stool samples should be analyzed as soon as possible after collection and if there is a need for storage samples should be frozen away (Nechvatal et al., 2008). Also, variability in the total DNA yield for the extraction methods may be partly due to intra-specimen variability, as faeces are heterogeneous biological materials (McOrist et al., 2002).

The DNA extraction method using lysis buffer L7a proved to be a method that is simple, sensitive, reproducible, rapid and cost effective. The cost for doing a DNA extraction (including the homemade spin columns by Borodina et al. (2003)) amounts to less than half the cost per a sample than in comparison to the commercially available QIAamp[®] DNA stool mini kit (Mieta et al., 2010). The recovered DNA is PCR ready and do not need the addition of any PCR facilitators such as bovine serum albumin (BSA) for amplification of the bacterial DNA (Kemp et al., 2006). This method was reported by Mieta et al. (2010) for isolating DNA from other bacterial pathogens such as *Salmonella*, *Shigella* and *Vibrio* species (Mieta et al., 2010), thus, adding to its attractiveness as a method for studying a wide range of bacterial pathogens in stool samples, since it requires only a single DNA extraction that is simple and cost-effective.

There is however, a need for a more in-depth study to validate the DNA extraction method against faecal matter from each animal source. This study only tested a small number of samples from each animal source to determine whether this DNA extraction method could be a viable option when studying animal stool samples.

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