RAPID DETECTION OF MICROBIAL CONTAMINATION IN GHANAIAN HERBAL MEDICINES BY PCR ANALYSIS

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SUMMARY

Background: There is widespread use of herbal medicines across the world and the need for regulatory measures to ensure their safety, efficacy and quality is therefore imperative. Conventional microbiological methods are used in carrying out quality control analysis of herbal medicines but these are often slow, may be less sensitive or specific and labour-intensive. In this study we investigated the ability to use Polymerase Chain Reaction (PCR) as a fast, accurate and inexpensive novel method to detect the presence of common pathogens in herbal medicines from Ghana.

Methods: We employed different DNA extraction techniques including TE buffer, boiling method for DNA extraction as well as commercially available DNA extraction kits from Qiagen, UK: Gentra Puregene Yeast/Bact. Kit and DNeasy[TM] Tissue Kit which is column based to identify *Escherichia coli, Staphylococcus aureus and Salmonella sp*. in herbal products from Ghana in local African shops on the UK market.

Results: The TE buffer and boiling methods did not yield any bacterial DNA, however both commercial kits yielded significant amounts of DNA. PCR was able to detect pathogens present in the samples directly. Escherichia coli could be detected at 10 cfu/ml whilst Staphylococcus aureus was detectable at a threshold of up to 10^3 cfu/ml when samples were enriched overnight. Salmonella sp. could not be detected in DNA samples extracted from herbal medicines.

Conclusion: We conclude that PCR and similar new molecular techniques such as Real Time PCR have the potential as rapid microbiological analytical techniques especially in busy clinical settings and for quality control of herbal medicines.

Key words: Herbal medicine, Microbial contamination, PCR, Ghana

INTRODUCTION

Traditional medicine is one of the oldest medicines used for centuries especially in many developing countries. Currently, WHO estimates that about 70-80% of the world's population, especially in developing countries, still depend on alternative or complimentary medicines including herbal remedies as their primary therapeutic.[1] The rising use of herbal medicines has seen an increase but there are issues related to quality and safety. The effect of quality in ensuring the safety and efficacy of herbal medicines has been extensively reviewed by several authors.[2,3] The quality of herbal medicines can be compromised due to the use of poor quality raw materials and the lack of effective controls thereby resulting in low quality of products. Herbal medicines are considered by some to be of a lower risk compared to orthodox medicines, but that notwithstanding, they cannot be said to be completely free from issues pertaining to toxicity and other adverse effects.[4]

Thus protocols and guidelines on safety, quality and toxicity testing have been developed by reputed international organizations[5,6]; the European Medicines Agency[7,8] and the European Food Safety authority[9] to help both the manufacturer and regulatory authorities to effectively assess and address these issues. Contaminants of herbal medicines are undesirable and potentially hazardous materials introduced during their handling at various stages of production. These contaminants have been grouped as chemical and biological contaminants, by the WHO10. Chemical contaminants are made up of heavy metals, residues from pesticides and agrochemicals and organic solvents used during processing.[10]

Common biological contaminants may include microorganisms - bacteria, fungi, some parasites and animals such as insects.

Microbial contamination can occur during the collection of raw materials and processing them into finished products due to poor quality control and hygiene practices during manufacture.[11] Faecal contamination may occur by the use of manure during cultivation of the plant, and also from the soil and contaminated water during processing, resulting in the introduction of pathogenic microorganisms such as Escherichia coli and Clostridium spp. into the products.

Regulation of herbal products is intended to give the consumer the assurance that the product is safe, of good quality and efficacious. Regulation of herbal medicines differs from country to country, the differences being primarily due to differences in cultural practices and the fact that herbal medicines are rarely studied scientifically.

In Ghana, the Food and Drugs Authority, (FDA) is responsible for the regulation of herbal medicines manufactured or imported into the country[12] and seeks to ensure that herbal medicines are safe, of good quality and effective to protect the consumer. The Quality Control Laboratory of the FDA is in charge of choosing analytical methods to assess the quality of a product. Currently, the laboratory uses the United States Pharmacopeia (USP) methods for Microbiological Examination of Non-sterile products and tests for specific organisms for the microbiological assessment of herbal medicines.

These standard microbiological methods are used to detect bacteria by employing morphological and biochemical methods to identify microorganisms[13] and also rely on the use of selective and or enrichment media to evaluate the microbiological quality of products.[14] Whereas, most bacteria can be cultured using standard microbiological methods, they are usually very slow, thus taking days to arrive at a conclusion. Other disadvantages include difficulty in identifying different species and strains of bacteria, specificity of methods to particular species and the ability to recover bacteria from a sample matrix to be analysed.[15]

To overcome these challenges, several other methods have been developed such as Polymerase Chain Reaction (PCR) to detect microorganisms in a wide range of samples by amplifying specific segments of DNA or genes in an organism.[16] The focus of this present study was to detect microbial contamination in herbal medicines collected from the Ghanaian market, targeted at children who may be particularly vulnerable because of their lack of fully developed immune system. The specific aim was to detect the presence of contaminants specifically *E. coli, S. aureus* and Salmonella sp. in herbal medicines by PCR using different DNA extraction protocols in order to reduce the time spent when using existing standard traditional microbiological methods.

MATERIALS AND METHODS
Seven different herbal medicines from different manufacturers originating from Ghana were purchased randomly from African shops in London, UK, processed and analysed in the Microbiology laboratory at the University of Greenwich using the following protocol:

Performing Total Viable Count on Herbal Samples
In order to establish the presence of microorganisms in the samples, a Total Aerobic Microbial Count was undertaken using USP method for Microbial Examination of Non-sterile Products: Microbial Enumeration Test was used.[17]

DNA Extraction Methods
DNA extraction using TE Buffer
TE buffer method described by Jimenez et al.[18] was used some modifications. 1ml of each sample was put into a sterile Eppendorf tube and then centrifuged at 10,000rpm for 10 minutes and the supernatant decanted leaving the cell pellets. 200µl of TE buffer (10mM TRIS-HCl, EDTA-1mM, pH 8.0) was added to the cell pellets. 6µl of 10 mg/ml of proteinase K solution (Promega, USA) and 0.5% Tween 20 were then added to the cell pellet solution and the mixture incubated at 55°C for 20 minutes to lyse cells and degrade cellular proteins. Samples were then transferred to the heating block (Digi-block^{TM} USA) and incubated at 95°C for 10 minutes. The resultant solution was centrifuged at 10,000rpm for 3mins and the supernatant decanted. Gel electrophoresis (0.8% agarose and 5 µg/ml of ethidium bromide) was run using 10µl of the supernatant to check the quality of the extracted DNA.

DNA Extraction by the Boiling Method
Boiling method also described by Jimenez et al.[18] was employed using1ml each of sample in a sterile Eppendorf tube and centrifuged at 10,000rpm for 10mins to pellet cells. The supernatant was decanted and discarded and 200µl of sterilized distilled water added to the cell pellets. This was incubated in a heating block at 100°C for 10mins. The solution was immediately transferred onto an ice bath for 3mins to cool and the resultant solution centrifuged at 10,000rpm for 3mins, the supernatant decanted and used for gel electrophoresis.

Use of DNA extraction Kits
Gentra Puregene Yeast/Bact. Kit and DNeasy^{TM} Tissue Kit both from Qiagen, UK was used in this experiment. 1ml of each sample was pipetted into Eppendorf tubes and centrifuged at 5000rpm for 10mins. Cell pellets were then collected and protocols for each of the kits

followed to extract DNA. For Gram positive bacteria, the cell pellets were frozen in liquid nitrogen and then thawed under room temperature for three consecutive times, to lyse the cell wall instead of using a Lysis Buffer. Gel electrophoresis was run on10µl of the resultant DNA.

Spiking and Enrichment of Samples to determine the Limit of Detection
Pure cultures of E. coli and S. aureus obtained from the Microbiology Laboratory with known number of colony forming units (cfu/ml) was spiked into 10ml of a sample containing no microorganism to obtain counts from 10 to 10^4 cfu/ml separately, for each organism. DNA was extracted from these spiked samples using Gentra Puregene Yeast/Bact. Kit. 1ml of the spiked samples was inoculated into 9ml of Tryptone Soy Broth medium (Oxoid, UK) and incubated overnight at 37^oC to enrich the cells and DNA extracted.

Polymerase Chain Reaction
Primers
Specific primers (Sigma-Aldrich, UK) targeted at the following genes: tuf with sequences as 5-TGGGAAGCGAAAATCCTG-3(forward),5-CAGTACAGGTAGACTTCTG-3(Reverse) for E. coli, catalase:5-TTCGAAGCCATTGAAAAAGG-3(forward),5-ACATCATCCGTTACGCCTTC-3(Reverse), for S. aureus and 16S RNA, 5-TGTTGTGGTTAATAACCGCA-3(Forward), 5-CACAAATCCATCTCTGGA-3(Reverse) for Salmonella were designed to amplify a 258bp, 641bp and 571bp fragments of the genes for *E. coli[19]* , *S. aureus* [20] and Salmonella. [21]were used in this study.

A stock primer solution of 100µM for each primer was prepared as per the manufacturer's instructions and kept in -20^oC from which 10µM of working solution was prepared.
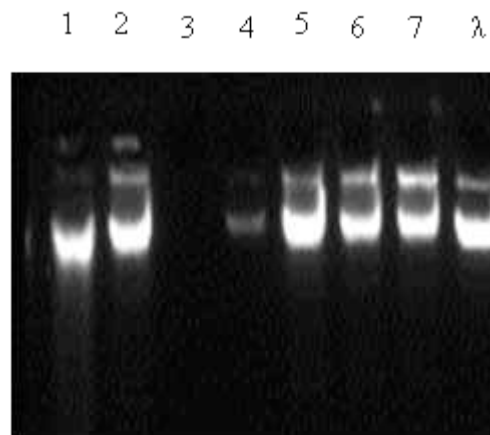
Polymerase Chain Reaction experiment
PCR was performed using the extracted DNA from each of the samples. A total reaction volume of 20µl was used, which contained 10µl Taq mix (Promega, USA), 1µl each of 10µM both forward and reverse primer solutions, 1mM $MgCl_2$, 3µl RNase/ DNase free water and 5µl DNA template extracted from samples. A negative control was performed by replacing 5µl of DNA template with water whiles a positive control contained 5µl of DNA extracted from pure cultures of each organism. PCR assays were performed in Gene-Pro PCR machine (Alpha Laboratories, UK).

The PCR progamme used consisted of initial denaturation, 94^oC for 5mins, 30 cycles for denaturation, 94^oC for 1min, annealing, 45^oC, 60^oC and 55^oC for *E. coli, S. aureus* and Salmonella sp. respectively for 30s, and extension, 72^oC for 1min and a final extension at 72^oC for 5mins. After PCR, 10µl of the PCR product was run on 1% gel. Bands were observed under UV light and photographs taken using Epichemi imager (UVP Bio imaging Systems, UK).

RESULTS
The presence of microorganisms in the samples was established by performing Total Aerobic Microbial Count the results of which showed that microbial contamination ranges from 0 - >3.0 x 10^6 cfu/ml. Only 1 out of the 7 samples analyzed had no microbial growth. This was therefore used to perform the spiking and enrichment test for determination of Limit of Detection.

For DNA extraction employing the different methods described above there was no DNA yield from the TE Buffer and Boiling methods (1 and 2). Employing DNA Extraction kits in method (3) however yielded good quality DNA, seen from the gel electrophoresis run using the DNA solution shown in (Figure 1.) below.
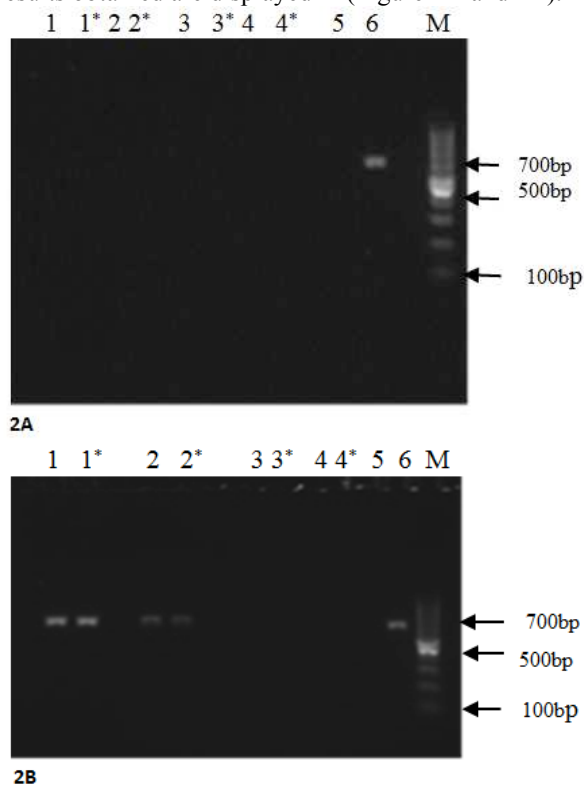

Figure 1 Photograph of agarose gel electrophoresis showing bands for genomic DNA extracted from the different herbal samples. Lanes: λ - Lamda Phage DNA used as marker, 1 - sample 1, 2 - sample 2, 3 – sample 3, 4 – sample 4, 5 – sample 5, 6 – sample 6,   7 – sample 7.

PCR assay results on samples showed bands for E. coli and S. aureus for 1 sample each. However no was band observed for Salmonella sp.

Bands were observed at 258bp for *E. coli* and 641bp for *S. aureus* as expected from the product sizes of the primers used and clearly observed in sample 2 in both gels.
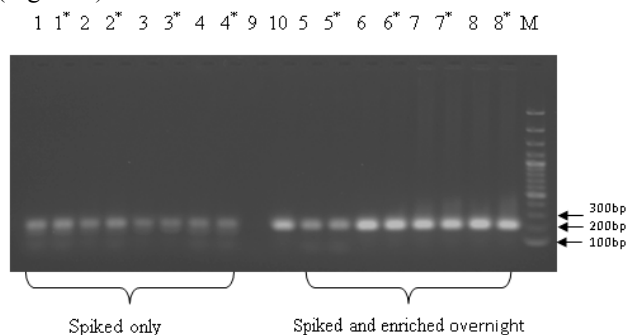
To find out the detection limits of theses microorganism drug samples were spiked with $10-10^4$ cfu/ml of E. coli and S. aureus separately and subsequent extraction of DNA and PCR performed. PCR analysis was carried out on both non-enriched and enriched samples to compare the effect of sample enrichment and any sample which did not have any growth on Tryptone Soy Agar was spiked with different concentrations of E. coli and S. aureus cells (separately). Concentrations of cells ranged from $10 – 104$ cfu/ml for both organisms. Overnight cultures were also prepared. DNA was extracted for all concentrations for both organisms. PCR assays were conducted using DNA extracted from the spiked samples, both enriched and non-enriched. The results obtained are displayed in (Figure 2A and 2B).

2A

2B

Figure 2 Photograghs of agarose gel electrophoresis of PCR products using S. aureus specific primers. 2A. (spiked only). Lanes: M 100bp DNA Marker, 1, 2 , 3, 4 – sample spiked with 10, 10^2, 10^3 and 10^4 respectively , 5 – negative control and 6 – positive control. 2B. (spiked and enriched overnight). Lanes: M –100bp DNA Marker, 1, 2 , 3, 4 – sample spiked with 10^4, 10^3, 10^2, and 10 respectively , 5 – negative control and 6 – positive control. (Lanes with * are duplicates).

Results for S. aureus showed that PCR could only detect samples spiked with 10^3 and 10^4 cfu/ml and enriched overnight. There was no detection for all non-enriched spiked samples and samples spiked with 10^2 and 10^3 cfu/ml and enriched overnight. Bands were observed between 600bp and 700bp which was to be expected (Figures 2A and 2B).

However, in case of E. coli PCR detected as low as 10cfu/ml from spiked samples for both enriched and non-enriched samples. The only difference between enriched and non-enriched samples was the band intensities, with the enriched samples being more intense (Figure 3).

Figure 3 Photogragh of agarose gel electrophoresis of PCR products for E.coli. Lanes: M –100bp DNA Marker, Spiked-only samples, lanes 1, 2, 3 and 4 represent spiking from 10^4- 10 cfu/ml respectively and for spiked and enriched, lanes 5 - 8 represent spiking from $10-10^4$ cfu/ml respectively. * represents duplicate samples Lane 9 – negative control and Lane 10 – positive control for E. coli DNA extracted from pure cultures.

DISCUSSION

Total Aerobic Microbial Count results show microbial contamination of herbal samples ranging from 0 - $>3.0x10^6$ cfu/ml. Of these, two samples had contamination levels above 10^5 cfu/ml, hence failing the WHO limit for aerobic bacteria which is 10^5 cfu/ml for liquids.[10] Similar work has been reported in Kaduna where they assessed contamination of herbal medicinal products marketed in Kaduna Metropolis in Nigeria. They observed total aerobic plate count on average of $≤5×10^7$cfu/g with a coefficient of determination showing that 28% of total samples studied had aerobic plate counts above the WHO limit.[22] Such high values are probably the result of manufacturers failing to follow rules of GMP during the manufacturing process.

Adopting different DNA extraction techniques is a very critical step since the yield and the quality of the extracted DNA has a direct bearing on PCR results.[23]

Therefore several DNA extraction techniques were adopted. The use of TE buffer and boiling method to extract bacterial genomic DNA from the samples yielded no DNA.

The use of commercially available DNA extraction kits, Gentra Puregene Yeast/Bact. Kit and DNeasy™ Tissue Kit (Qiagen, UK) were used to extract the DNA from samples reduced the preparation time needed for reagents in conventional DNA extraction methods. It has also been shown that commercially available kits yield good quality amplifiable products compared to standard methods and in-house extraction methods.[24] Gram positive bacteria pellets were pre-treated by freeze thawing cycles in liquid nitrogen and at room temperature respectively enabled the effective lysis of the cell wall instead of a Lysis buffer thus saving time and money. Gentra Puregene Yeast/Bact. Kit was used for extracting DNA from the freeze thawed pellets and agarose gel electrophoresis showed DNA indicating that the freeze thaw method is effective for lyses of the cell walls of Gram positive bacteria.

Gel electrophoresis photographs of PCR showed that both E. coli and S. aureus, DNA were amplified in sample 2 where expected product size of 258bp and 641bp were observed for $E. coli$[19] and $S. aureus$,[20] respectively. Salmonella sp. was not detected following DNA extraction from samples and was thus probably absent or of low quantity to be detected.

For limit of detection experiment in case of S. aureus, bands were only observed for overnight enriched samples with cell concentrations of 10^3 and 10^4 cfu/ml of sample (Figure 2B) but not for non-enriched samples spiked with 10 to 10^4 cfu/ml (Figure 2A) and enriched samples with cell concentrations of 10 and 10^2 cfu/ml (Figure 2B). In another study it was observed that PCR was able to detect S. aureus at 10^2 to 10^7 cfu/ml using the same primers in milk products. Inability to detect S. aureus in non-enriched and lower sample concentrations in this study may be due to the medium, in this case herbal product matrix rather than milk.[20]

Gel electrophoresis results for PCR products for E. coli (Fig 3) for both enriched and non-enriched samples showed bands for all cell concentrations thus indicating that for E. coli, PCR is able to detect as low as 10 cfu/ml in herbal medicinal samples. This has been demonstrated elsewhere by others where it was shown that PCR is able to detect E. coli cells between $10 – 10^2$ cfu/g in soil samples.[25]

CONCLUSIONS
We have demonstrated that PCR is able to detect the presence of E. coli and S. aureus in liquid herbal medicinal products. We believe that PCR analysis is a rapid and reliable method with potential time-saving advantages for clinical practice and quality control of herbal medicinal products but this potential merits further exploration.

Despite its usefulness, conventional PCR is however a qualitative method, therefore we also suggest further experiments to explore the use of real time PCR to enable both qualitative and quantitative determination of microbial load in herbal medicines for public health monitoring.

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