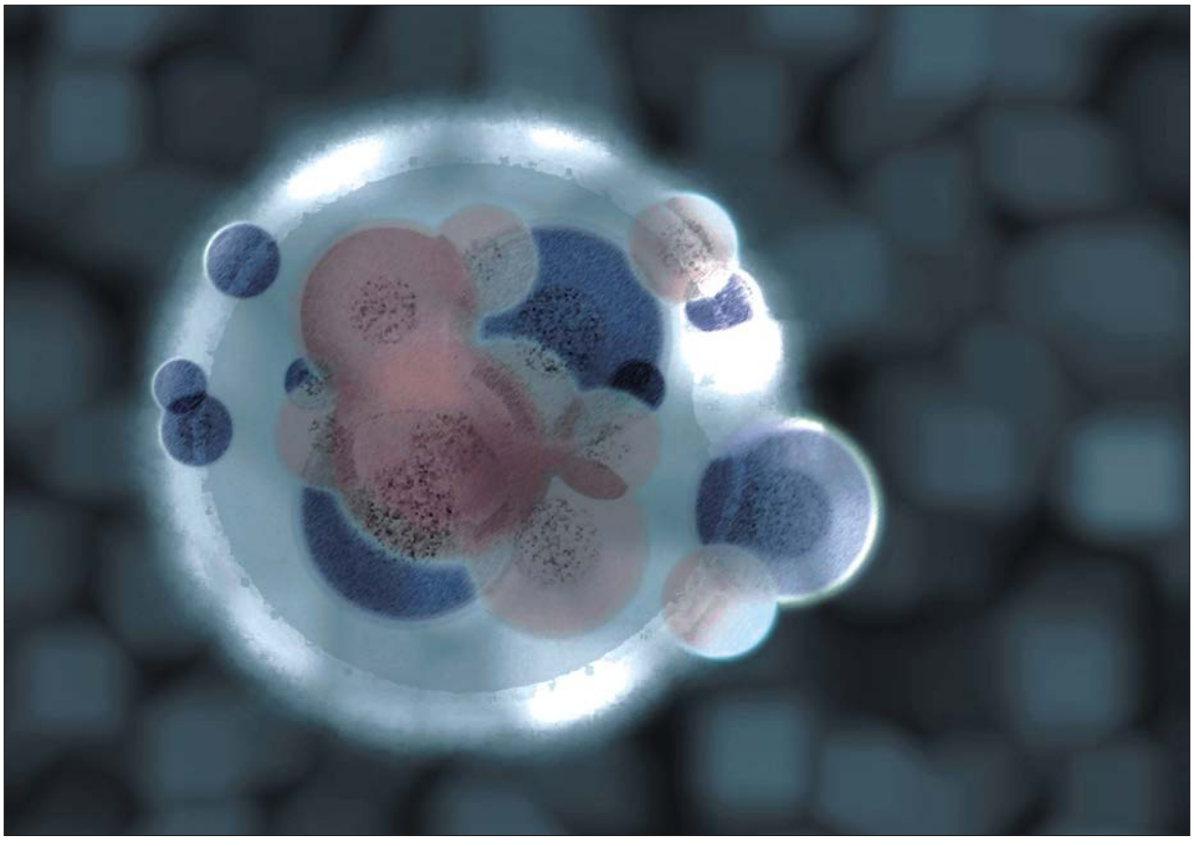

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PCR-Based Detection of Low Levels of *Staphylococcus aureus* Contamination in Pharmaceutical Preparations

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Abstract: Polymerase Chain Reaction (PCR) was performed to verify the utility of this technique in detecting low levels of microbial contamination in quality control of pharmaceutical products. Universal and specific primers were applied to identify *Staphylococcus aureus* at both genus and species levels, as one of the objectionable microorgamisms in pharmaceuticals especially topical products. Samples were deliberately inoculated with a defined number of bacterial cells and subsequently exposed to mild lysis. The crude lysate was subjected to PCR amplification. Agarose gel electrophoresis revealed amplified fragments as predicted, with no interference from other bacterial strains included in the study. The sensitivity of the assay was about 10^2 cfu mL^{-1} which improved to 1 cfu mL^{-1} after an overnight preenrichment. Combination of this simple lysis and rapid PCR protocol provided a highly sensitive and practical approach in detecting trace amounts of *Staphylococcus aureus* contamination in pharmaceutical preparations. In contrast to conventional culturing methods that require a mean of 5-6 days for identification of microorganisms, the entire mentioned PCR assay lasted about 1-2 h.

Key words: PCR, *Staphylococcus aureus*, contamination, pharmaceuticals

INTRODUCTION

Microbial contamination in pharmaceutical products could result in a diminished shelf life by compromising product integrity or present potential health hazard to the consumers. Microbiological specifications for pharmaceutical products are principally based upon the chemical nature of the product ingredients and the intended use of a given formulation (Mestrandrea, 1997). In non-sterile pharmaceutical preparations, absence of pathogenic bacteria such as *Staphylococcus aureus* (*S. aureus*), is necessary. To cope with this requirement, laboratory techniques have long relied on conventional methods i.e., culturing methods, directly observing phenotypic characteristics of the colonies and biochemical tests. Although these methods have been applied in many quality control systems, they are time-consuming, non-specific to some extent and often give rise to delayed delivery.

To provide a more comprehensive view of the prevalence of potential contaminants, establishment of a rapid, sensitive and cost-effective method is imperative. Numerous assays have been carried out on rapid methods dealing with food samples (Hill, 1996; Lindqvist *et al*., 1997) and clinical analysis (Martineau *et al*., 1998; Mason *et al*., 2001), while few studies have been devoted to application of rapid technologies in elucidating the presence of microorgamisms in pharmaceutical preparations. This study was aimed at precise and quick tracing of *S. aureus* at species level in pharmaceutical products, using a PCR assay with no need for further manipulation steps.

MATERIALS AND METHODS

Microorganisms: Two *Staphylococcus aureus* strains, ATCC 6538P and ATCC 29737 were used in this study. The specificity of the DNA-based test for identification of

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Staphylococcus aureus was verified in genus level by using several Gram-positive and Gram-negative bacteria (*Streptococcus sanguis* CIP 53.158, *Escherichia coli* ATCC 8739, *Salmonella typhimurium* (clinical isolate), *Lactobacillus plantarium* (clinical isolate), *Pseudomonas aeruginosa* ATCC 9027 and *Bacillus subtilis* ATCC 6633) and in species level by using a number of *Staphylococcus* sp. including *Staphylococcus epidermidis* ATCC 14990, *Staphylococcus warneri* ATCC 27836, *Staphylococcus haemolyticus* ATCC 29970, *Staphylococcus luteus* ATCC 29070, *Staphylococcus scuiri* ATCC 29062, *Staphylococcus saprophyticus* ATCC 15305 and *Staphylococcus xylosus* ATCC 29971.

Sample preparation: A topical lotion was selected as the test pharmaceutical product. The bacterial inocula were prepared by suspending overnight colonies from Mueller-Hinton agar media in 0.9% saline. The inocula were adjusted photometrically at 600 nm to a cell density equivalent to approximately 0.5 McFarland standard (1.5×10^8 cfu mL^{-1}). Test samples were inoculated with each bacterial suspension to a final concentration of about 1×10^6 cfu mL^{-1}. This bacterial concentration was verified by serial dilution and plate count method.

DNA extraction and PCR amplification: From artificially contaminated samples, 100 μL aliquots were added to 200 μL of lysis buffer (Tris 10 mM, EDTA 1 mM, Tween 20 (0.5 %) and 3 μL of Proteinase K) at pH 8.0. The samples were incubated at 30-35°C for 20 min and afterwards boiled in 95°C for 10 min. Two pairs of primers, namely universal primers (5'-GGA GGA AGG TGG GGA TGA CG-3' and 5'-ATG GTG TGA CGG GCG GTG TG-3') and *S. aureus* specific primers [S1 (5'-AAT CTT TGT CGG TAC ACG ATA TTC TTC ACG-3') and S2 (5'-CGT AAT GAG ATT TCA GTA GAT AAT ACA ACA-3')] were synthesized by MWG-BIOTECH AG, Germany. The universal primers were derived from highly conserved regions of the bacterial 16S rRNA gene, providing a positive control because they can amplify a 241 bp product from any bacterial species. The specific primers code for a 108 bp product.

The reaction mixture (25 μL) consisted of 0.4 μM (each) forward and reverse universal or specific primers, 400 μM (each) four deoxynucleoside triphosphate, 10 μL of the lysate as DNA template, 1.25 U *Taq* DNA polymerase (Roche, Germany) and the manufacturer's supplied buffer (10 mM Tris/HCl, 50 mM KCl, 2.5 mM $MgCl_2$). Control reactions containing double distilled water instead of DNA templates were also included throughout the PCR assay to avoid false positive results. Universal primers were used to verify the efficiency of bacterial lysis method, the PCR assay and absence of PCR inhibitory substances.

PCR was conducted in the thermocycler (Techne flexigene, UK) with initial denaturation at 96°C for 3 min and 40 cycles of denaturation at 95°C for 1 sec and annealing-extension at 55°C for 30 sec.

Agarose gel electrophoresis was carried out at 100 V for 30 min using 1.5% ME agarose gel (USB™, Cleveland Ohio) containing 0.5 μg of ethidium bromide per mL. The gel was photographed using BioDoc-It™ system (UVP, USA).

Sensitivity testing: For the purpose of estimating minimum bacterial cell detection limit in presence of other bacterial contaminants, sensitivity testing was carried out with *S. aureus* specific primers. A bacterial suspension adjusted photometrically at 600 nm to approximately 1.5×10^8 cfu mL^{-1} was diluted in 0.9% saline to prepare a series of 10 fold decreasing concentrations of *S. aureus*. The pharmaceutical samples were inoculated separately with above mentioned dilutions of *S. aureus* to contain about 10^6 to 1-2 cfu mL^{-1}. Each sample was simultaneously spiked with *E. coli* and *P. aeruginosa* to a final concentration of about 10^6 cfu mL^{-1}.

Five milliliter of each spiked sample was aseptically transferred to 50 mL of preenrichment medium (Soybean-Casein Digest broth, Merck), mixed and incubated at 30-35°C for 24 h.

DNA extraction and PCR amplification was performed on the spiked samples immediately before incubation and after an overnight enrichment.

RESULTS

As shown in Fig. 1 the expected 241 bp DNA fragment was successfully amplified with universal primers for all 9 staphylococcal (Fig. 1a) and 6 non-staphylococcal species tested (Fig. 1b). These results proved the efficacy of simple lysis and PCR assay used in our experiments and ensured that the pharmaceutical samples ingredients posed no adverse effect to PCR detection.

The 108 bp DNA fragment was amplified using specific primers and *S. aureus* target DNA. No amplification products were observed using template DNA from other strains nor with negative water controls (Fig. 2).

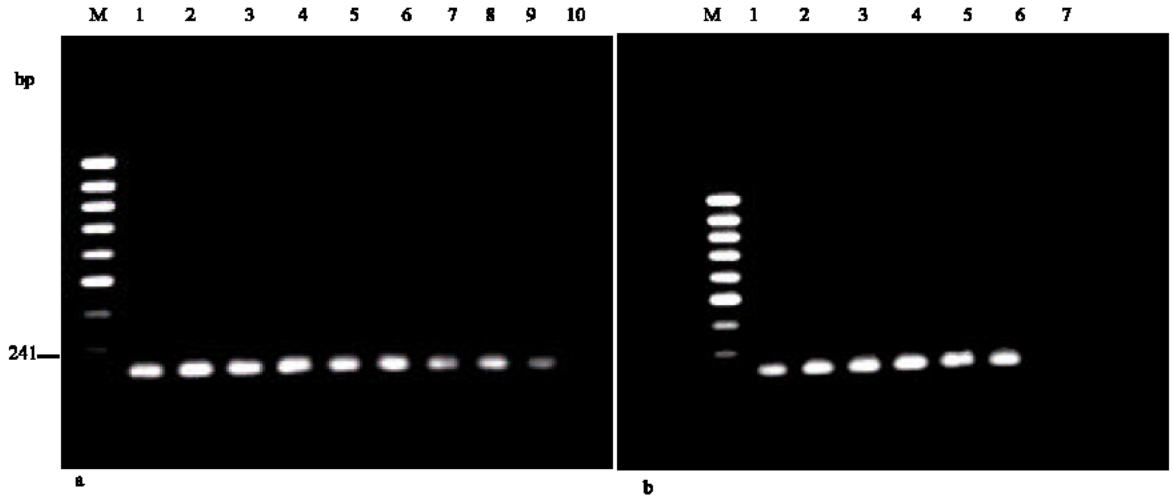

Fig. 1: PCR assay of pharmaceutical samples contaminated with 10^6 cfu mL^{-1} of a) a Staphylococcal; or b) a non-Staphylococcal strain, using universal primers. DNA templates were obtained from the product contaminated with a) 1, *S. aureus* ATCC 6538P; 2, *S. aureus* ATCC 29737; 3, *S. epidermidis* ATCC 14990; 4, *S. warneri* ATCC 27836; 5, *S. haemolyticus* ATCC 29970; 6, *S. luteus* ATCC 29070; 7, *S. saprophyticus* ATCC 15305; 8, *S. xylosus* ATCC 29971; 9, *S. scuiri* ATCC 29062; 10, negative control (water blank); or b) 1, *Streptococcus sanguis* CIP 53.158; 2, *Escherichia coli* ATCC 8739; 3, *Salmonella typhimurium* (clinical isolate); 4, *Pseudomonas aeruginosa* ATCC 9027; 5, *Bacillus subtilis* ATCC 6633; 6, *Lactobacillus plantarium* (clinical isolate); 7, negative control (water blank); M, molecular size marker

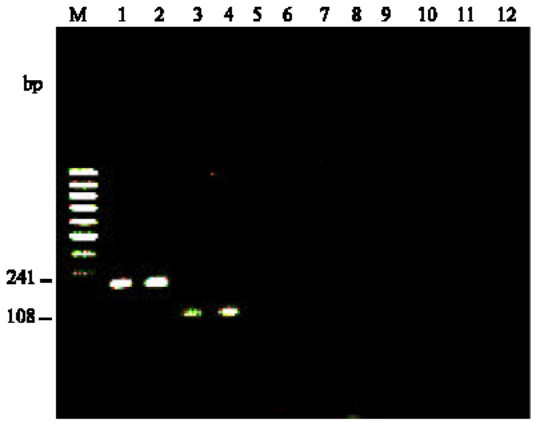

Fig. 2: Determination of PCR specificity for detection of *S. aureus* in pharmaceutical samples contaminated with the following test strains: Lanes 1 and 2 show fragments amplified with universal primers and template DNA from *S. aureus* ATCC 6538P and 29737, respectively; Lanes 3-11 show the results of PCR using *S. aureus* specific primers and DNA templates from: 3, *S. aureus* ATCC 6538P; 4, *S. aureus* ATCC 29737; 5, *S. epidermidis* ATCC 14990; 6, *S. warneri* ATCC 27836; 7, *S. haemolyticus* ATCC 29970; 8, *S. luteus* ATCC 29070; 9, *S. saprophyticus* ATCC 15305; 10, *S. xylosus* ATCC 29971; 11, *S. scuiri* ATCC 29062; Lane 12, negative control (water blank) and M, molecular size marker

The sensitivity of the assay was determined to be about 10^2 cfu mL^{-1} which further improved to about 1 cfu mL^{-1} after 24 h preenrichment (Fig. 3a and b). Although 40 cycles were performed in all experiments, 30 cycles showed to be just as effective to detect *S. aureus* at species level in pharmaceutical products.

No additional fragment was amplified with either *E. coli* or *P. aeruginosa* DNA templates present at the same time in the spiked samples (Fig. 3a and b).

DISCUSSION

To cope with required microbial limit tests, there is always a need for a rapid, sensitive and efficient method. The conventional methods for detection of microbial contamination in pharmaceutical products are generally based on culture in selective media, microscopical examination of the suspected colonies and biochemical tests and therefore time-consuming and non-specific.

In recent years attempts have been focused on specific, sensitive and rapid methods because available documents are still insufficient to integrate rapid methods in identification of microbial contaminations. Some of these methods like bacterial fingerprinting require a high quantity of intact and pure genomic DNA (Tyler *et al*., 1997). Use of purified DNA is also recommended for repetitive PCR and random polymorphic DNA analysis (Tyler *et al*., 1997). Some methods such as hybridization test for rRNA (Davis and Fuller, 1991) and an enzymatic

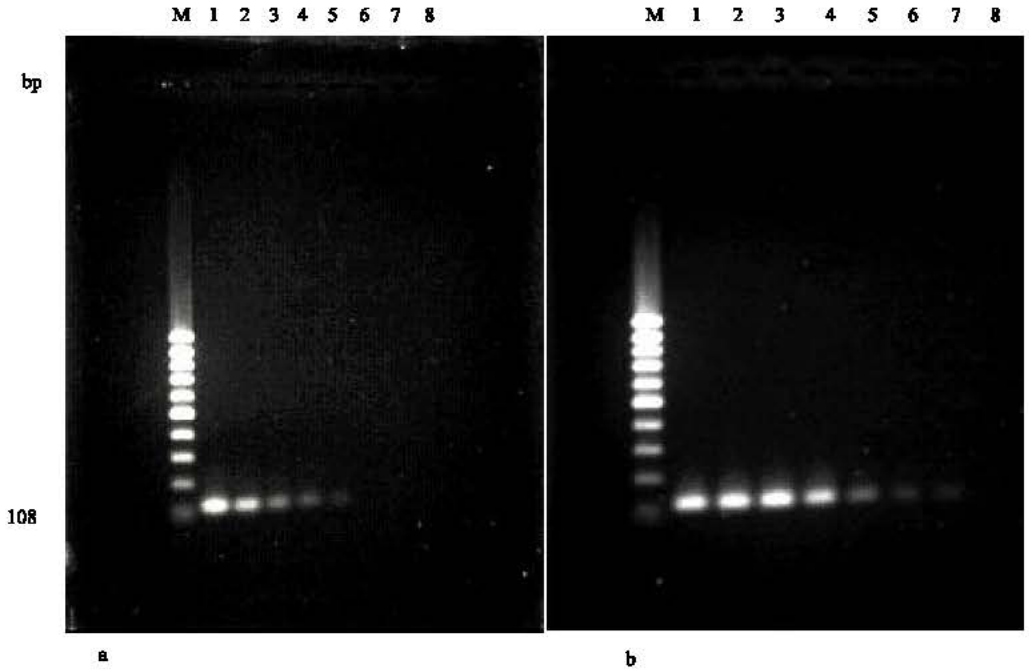

Fig. 3: Determination of PCR sensitivity for detection of *S. aureus* in pharmaceutical samples simultaneously contaminated with *E. coli* and *P. aeruginosa* (10^6 cfu mL^{-1}) using specific primers; a) prior and b) after an overnight preenrichment; Lanes 1-7 correspond to the samples containing 10^6 to 10^0 cfu mL^{-1} of *S. aureus*; lane 8, negative control containing *E. coli* and *P. aeruginosa*; lane M, molecular size marker

test for the detection of thermonuclease (Ratner and Stratton, 1985) have also been investigated, with the former yielding false positive results and the latter lacking sensitivity.

The present study is directed towards the evaluation of a simple lysis protocol coupled with a speedy PCR system for detection of low numbers of *S. aureus* in pharmaceutical products. The extraction method applied, using lysis buffer containing proteinase K, but no PCR inhibitory reagents and subsequent boiling was adequate to acquire the DNA-template for the next step. This obviated the need for further purification with hazardous and PCR inhibitory substances (i.e., phenol and chloroform). Some experiments used lysostaphin or lyzozyme for DNA extraction, which are expensive or SDS which necessitates various purification steps because of its PCR inhibitory nature.

In the PCR assay of our experiment the distinctive 241 bp fragment obtained with universal primers and all bacterial DNA templates, indicated the efficiency of the extraction method and primers. Amplification of the 108 bp fragment with specific primers using *S. aureus* template DNA and negative results with other Staphylococcal species proved the specificity of the assay.

Some studies have used primers to amplify a region from 16S rRNA gene sequence (Saruta *et al*., 1995; Jimenez *et al*., 2000) however this region is too similar among bacteria to allow identification of strains at species level.

In this study the Minimum Detection Limit (MDL) of *S. aureus* in pharmaceutical samples using 10 μL of the sample lysate without any prior enrichment was 10^2 cfu mL^{-1}. This level of detection has not been previously reported in pharmaceutical samples.

As a major drawback of PCR is its lack of discrimination between viable and non-viable cells, we integrated a preenrichment step into our study. After a 24 h preenrichment the number of cells increased and the MDL was lowered to 1 cfu mL^{-1} (Fig. 3a and b). The positive PCR results obtained for the bacterial concentration of about 10^1 and 10^0 cfu mL^{-1} and intensification of other amplicons following enrichment could be expected, since bacteria would multiply exponentially within 24 h.

While standard identification methods require a mean of several days, in this study with the virtue of quick lysis, specific primers and PCR protocol, *S. aureus* contaminations of about 1-2 cfu mL^{-1} in pharmaceutical products were identified to the species level.

ACKNOWLEDGMENTS

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