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Molecular detection of bacterial indicators in cosmetic/ pharmaceuticals and raw materials

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PCR assays were compared with standard microbiological methods for rapid detection of the United States Pharmacopoeia (USP) bacterial indicators in artificially contaminated samples of raw materials and cosmetic/pharmaceutical products. DNA primers containing the specific sequences of the *uidA* gene of the β-glucuronidase enzyme for *Escherichia coli*, the membrane lipoprotein gene *oprL* for *Pseudomonas aeruginosa*, and the 16S ribosomal gene for *Staphylococcus aureus* were used for detection in the PCR reaction. Contaminated samples were incubated for 24 h at 35°C. After incubation in broth media with and without 4% Tween 20, samples were streaked on selective growth media. After 5–6 days, all microbial indicators were morphologically and biochemically identified using standard methods while detection and identification by the PCR-based assays was completed within 27–30 h. Rapid PCR detection of *E. coli*, *S. aureus*, and *P. aeruginosa* will allow a faster quality evaluation and release of raw materials and cosmetic/pharmaceutical products sensitive to microbial contamination.

Keywords: PCR; USP; bacterial indicators; rapid detection; cosmetic/pharmaceuticals; quality control; *Staphylococcus aureus*; *Escherichia coli*; *Pseudomonas aeruginosa*

Introduction

Quality control evaluation of non-sterile cosmetic/ pharmaceutical formulations requires microbiological analysis of raw materials and finished products to monitor the safety of a given sample [8]. Standard microbiological methods require enumeration, isolation, and identification of microbial contaminants detected in finished products [9]. Based upon the pure-culture principle, these methods are time consuming, expensive, and require large amounts of media.

Development and application of rapid methods for food testing and clinical analysis have resulted in faster quality evaluation of foods and rapid diagnosis of pathogens [2,5]. However, rapid method development and application for cosmetic/pharmaceutical quality control have been slow. Rapid methods for the analysis of the microbial content of fluoride and Triclosan dentifrices using ATP bioluminescence have been developed and validated for the 24–30 h detection of contamination by bacteria, yeasts, and molds [4,6].

In addition to the microbial content, microbiological analysis must also determine the safety of a given product by the absence of certain types of microorganisms which can be considered a hazard to consumers [9]. For this purpose, the United States Pharmacopoeia (USP) Microbial Limits Test requires the absence of four different bacterial indicators: *Salmonella typhimurium*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli*. One of these indicators, *S. typhimurium*, has been detected and identified within 30 h in samples of raw materials and finished products using a commercial PCR-based system [7]. However, no PCR assay has been developed and validated for the detection and identification of the other three USP bacterial indicators for pathogen screening of non-sterile cosmetic/ pharmaceutical products.

The purpose of this study was to develop and validate PCR-based assays for rapid detection of: *S. aureus*, *E. coli*, and *P. aeruginosa* in those raw materials that are microbially sensitive and non-sterile cosmetic/ pharmaceutical finished products.

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Materials and methods

Standard enrichment/selective method

Ten-gram or ten-milliliter samples of raw materials and finished products were aseptically added to 500 ml of sterile lactose broth or Trypticase Soy Broth (TSB) for raw materials and lactose broth or TSB containing 4% Tween 20 for finished products. After thorough mixing, each of these enrichment broths was inoculated by transferring 1 ml of a 10^{-7} dilution of 24-h broth cultures of *S. aureus* ATCC 6538, *E. coli* ATCC 8739, or *P. aeruginosa* ATCC 9027. Inoculated samples were incubated for 24 h at 35°C. Lactose broth enrichments were streaked on Eosine Methylene Blue agar (EMB) and MacConkey agar. Inoculated TSB enrichment broths were steaked on Pseudomonas Isolation Agar (PIA), Vogel–Johnson Agar (VJ), and Mannitol-Salt (MS) agar. After a 24–48 h incubation at 35°C, colonies were streaked onto sterile plates of Trypticase Soy Agar (TSA) for isolation of pure cultures. TSA plates were incubated for 18–24 h and cells from pure cultures were gram stained. Biochemical identification of individual colonies was performed using the VITEK Jr Automatic Identification System and the ATB Identification System (bioMerieux, Hazelwood, MO, USA).

DNA extraction from artificially contaminated samples

After a 24-h incubation, a 10-μl aliquot of each broth was added to 200 μl of lysis buffer containing TRIS (1 mM)-EDTA (0.1 mM), 3 μl of a 20-mg ml^{-1} Proteinase K solution, and 0.5% Tween 20 at pH 8.0. These samples were incubated for 20 min at 35°C to lyse cells and degrade cellular proteins. Samples were incubated for an additional 10 min at 95°C to complete lysis and destroy the protease.

A second lysis treatment was analyzed. One-milliliter aliquots of artificially contaminated samples were centrifuged for 10 min at 10 000 × *g*. Pellets were suspended in 1 ml of sterile deionized water. Samples were boiled for 10 min followed by centrifugation at 10 000 × *g*. A 4-μl aliquot of the supernatant fluid was used for the PCR reaction.

PCR amplification of artificially contaminated samples

For each of the *E. coli*, *S. aureus*, and *P. aeruginosa* samples, 4 μl of lysate were transferred into a PCR tube containing 94 μl of sterile water, 1 μM of each primer, and Ready-To-Go PCR beads (pH 9.0) (Pharmacia Biotech, New Brunswick, NJ, USA).

E. coli was detected by using primers UAL-754 and UAL-900 [1] to amplify a 147-bp DNA region of the *uidA* gene that specifies for the β-**d**-glucuronidase enzyme. To detect *P. aeruginosa*, PAL-1 and PAL-1 DNA primers were used to amplify a 504-bp fragment that encodes for the membrane lipoprotein *orpL* [3]. *S. aureus* was detected using primers SA-1 *and* SA-2 which encode for a 273-bp fragment from genomic 16S rRNA genes [10]. Commercially synthesized DNA primers were used (Genosys Biotechnologies, The Woodlands, TX, USA).

Sample tubes were loaded into a thermocycler (Perkin Elmer 9600, Foster City, CA, USA) and the PCR reaction was initiated. The reaction conditions for the PCR reactions have been previously described [1,3,10]. Amplified samples were then analyzed by agarose gel electrophoresis using 2.0% SeaKemR Gold Reliant agarose gels which had been prestained with ethidium bromide (FMC, Rockland, ME, USA). Photographs were taken using a Foto/Phoresis UV Documentation System (Fotodyne, Hartland, WI, USA).

Results and discussion

To validate the PCR assay against standard methods, the following microbially sensitive raw materials and finished products were analyzed: neobee oil, simethicone, carboxymethylcellulose, fluoride dentifrice, sleeping tablets, medicated skin cream, ointment, antiflatulent liquid drops, sodium alginate, raspberry flavor, hydroxymethylcellulose, xantham gum, silica calcinate, denture adhesive, gel dentifrice, baking soda dentifrice, medicated dentifrice-A, medicated dentifrice-B, gel dentifrice, guar gum, starch, lactose monohydrate, diatomaceous earth, and laxative tablets. Conventional methods for detecting pathogens in samples involve pre-enrichment of the samples in order to allow growth of any microorganisms present. The incubated enrichment broth is then streaked onto selective/differential agar for isolation of the target microorganisms. After incubation, representative bacterial colonies were selected and transferred to TSA for morphological and biochemical identification. *E. coli, S. aureus*, and *P. aeruginosa* were isolated and biochemically identified from all the artificially contaminated samples. The biochemical profile numbers for the different bacterial colonies identified as *E. coli* were 74073730003, 74077730003 for the ATB identification system and 30017213600, 30017213640 for the Vitek Jr identification system. The Vitek Jr biochemical profile numbers for the different bacterial colonies identified as *P. aeruginosa* were the following: 34301000110, 34307000110, 34303000110, 34717000110, 34703000100, 34707000100, 34713000110, 64673065073, 60763067073, and 60363065073. The biochemical profile numbers for the colonies identified as *S. aureus* were: 77427060014, 734250600 for the Vitek Jr identification system, and 267316700, 06731660 for the ATB identification system. However, isolation and final identification of the microbial colonies as *E. coli*, *P. aeruginosa*, and *S. aureus* were completed after 4–5 days (data not shown).

The specificity of the different DNA primers used in the PCR-based assays has been reported [1,3,10]. To determine the optimal number of Ready-To-Go PCR beads necessary for optimal PCR amplification, different numbers of beads were analyzed with DNA samples for each bacterial indicator. The beads provide the necessary reagents for the PCR reaction in a convenient, ambient temperature-stable form. The beads contain buffer, nucleotides, and the *Taq* polymerase. Once the beads were rehydrated, the only reagents needed were the sample lysate and the specific primers. Optimal PCR amplification for *E. coli* and *P. aeruginosa* required a minimum of two beads while *S. aureus* PCR amplification required four beads. To determine the sensitivity of the PCR assay, serial dilutions of the microbial indicators were analyzed. The minimum detectable level of cells for *E. coli*, *P. aeruginosa*, and *S. aureus* were 10^5 cells ml^{-1}.

Sample preparation prior to the PCR reaction is a critical step for optimization of any PCR-based assay. Extraction of microbial DNA from the contaminated sample mixture will provide the template that will be used by the *Taq* DNA polymerase enzyme to amplify the specific DNA target. To determine the efficiency of the sample preparation on PCR detection of the three bacterial strains, two procedures were evaluated. For *E. coli* and *P. aeruginosa*, samples were treated with a lysis buffer prior to the PCR reaction. This treatment was as effective as boiling the samples for 10 min. However, PCR detection of *S. aureus* contamination was not possible without the use of a lysis buffer treatment. Therefore, the use of a lysis buffer treatment was incorporated as part of the sample preparation prior to PCR analysis. The lysis buffer containing Proteinase K, Tween 20, and TRIS (1 mm)-EDTA (0.1 mM) at pH 8.0 allowed gentle lysis of bacterial cells present in the sample mixture resulting in optimal amplification of specific bacterial DNA sequences.

The test results of PCR amplification of some of the samples inoculated with *E. coli*, *P. aeruginosa*, and *S. aureus* are shown in Figure 1. After 24-h bacterial growth, the 147-bp DNA fragment specific for *E. coli* was found in samples of artificially contaminated raw materials and finished products (Figure 1a). Gel electrophoresis results

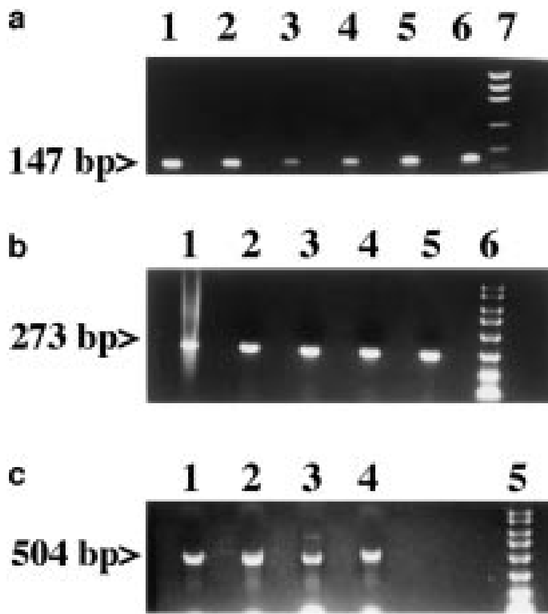

Figure 1 Gel electrophoresis of PCR amplification of raw materials and products contaminated with bacterial indicators. (a) *E. coli*. Lanes 1: fluoride dentifrice; 2: medicated cream; 3: sleeping tablets; 4: ointment; 5: antiflatulent liquid; 6: sodium alginate; 7: markers (base pairs): 2000, 1200, 800, 400, 200. (b) *S. aureus*. Lanes 1: gel dentifrice; 2: medicated dentifrice-A; 3: baking soda dentifrice; 4: fluoride dentifrice; 5: gel dentifrice; 6: markers (base pairs): 2000, 1500, 1000, 750, 500, 300, 150, 50. (c) *P. aeruginosa*. Lanes 1: baking soda dentifrice; 2: gel dentifrice; 3: medicated dentifrice-B; 4: fluoride dentifrice; 5: markers (base pairs): 2000, 1500, 1000, 750, 500, 300, 150, 50.

showing the 273-bp DNA fragment specific for *S. aureus* are shown in Figure 1b. The *S. aureus* DNA fragment was present in deliberately contaminated samples of all five different dentifrice formulations. Similar results were found with samples contaminated with *P. aeruginosa* (Figure 1c). The distinct 504-bp *P. aeruginosa* DNA fragment was detected in all the contaminated samples of the raw materials and finished products. None of the different tested types of finished products (creams, tablets, emulsions, and dentifrices) and raw materials (gums, powders, and oils) inhibited the PCR reaction. This demonstrated that the different chemical composition of the raw materials and finished products did not interfere with the PCR reaction. Previous studies of PCR analysis of foods and clinical samples required stringent conditions for sample preparation due to the presence of inhibitory substances [2,5].

Both the PCR and the standard enrichment test method detected *E. coli*, *P. aeruginosa*, and *S. aureus* in all of the deliberately contaminated samples. The time to complete the PCR assay including sample preparation and PCR amplification of the specific DNA bacterial targets was 27–30 h. Standard plating methods required 4–5 days for the bacteria to be isolated, purified, and biochemically identified. A PCR-based assay to detect the presence of the other USP indicator *Salmonella typhimurium* in raw materials and finished products had previously been reported [7]. However, in that study, all reagents for the PCR reaction were supplied in a tablet formulation as part of a commercial kit, the BAX™. In this study, the sample lysate and the PCR primers were added to the Ready-To-Go PCR beads. The use of the Ready-To-Go PCR beads minimized sample handling and preparation. Nevertheless, it might be possible to combine all of the reagents needed by developing a specific tablet for each USP indicator microorganism or to develop a specific multiplex PCR for the simultaneous amplification of the four USP bacterial indicators.

In summary, rapid and simple PCR assays were developed and validated for detection of the USP bacterial indicators: *E. coli*, *P. aeruginosa*, and *S. aureus*. These data and a previous study have shown that PCR technology permits a 27 to 30-h detection of the four USP bacterial indicators, allowing rapid evaluation of raw materials sensitive to microbial contamination and of finished products. Rapid quality evaluation aids the optimization of product manufacturing, quality control, and release in cosmetic/pharmaceutical environments.

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