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MALDI-TOF MS BIOTYPER AND POLYMERASE CHAIN REACTION FOR RAPID IDENTIFICATION OF *STAPHYLOCOCCUS AUREUS* AND *PSEUDOMONAS AERUGINOSA* IN NON-STERILE PHARMACEUTICAL PREPARATIONS

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ABSTRACT: Absence of pathogenic bacteria is a must for passing the microbiological quality control inspection of non-sterile pharmaceutical products. The identification of microbial contaminants by conventional methods is time-consuming and labor-intensive. The overall aim of the study was to test non-conventional methods as polymerase chain reaction (PCR) and Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectroscopy Biotyper (MALDI-TOF) for rapid identification of *Pseudomonas aeruginosa* and *Staphylococcus aureus* in topical pharmaceutical preparations. Four different topical preparations and three raw materials were artificially inoculated with tested microorganisms, at an initial cell count of 10º CFU/ml, and were overnight pre-enriched in Tryptic soy broth (TSB). MALDI-TOF was tested for identification of target microorganisms using different sample preparation methods. Duplex PCR targeting *opr*L (*Pseudomonas aeruginosa*) and mRNA nuclease gene (*Staphylococcus aureus*) was also tested. *Pseudomonas aeruginosa* and *Staphylococcus aureus* were identified within 18 - 37 h using MALDI-TOF, according to the sample preparation method, and 27 h using duplex PCR; compared to 48 - 72 h for the identification using conventional microbiological methods. Our results revealed that both PCR and MALDI-TOF were sensitive and specific to target microorganisms; MALDI-TOF proved to be a precise, cheap, rapid and high throughput screening method, compared to PCR and conventional methods.

INTRODUCTION: Microbiological assessment of non-sterile products and raw materials is one of the control stages to ensure the quality and safety of finished products. Microbial contamination of pharmaceutical products can reduce or even eliminate the therapeutic effect of drugs by: changing the chemical, physical and organoleptic properties of the drugs or changing the contents of active ingredients.

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Besides, it can cause drug-induced infections; convert drugs to toxic products, and making them hazardous to health [1]. A previous study reported *Serratia marcescens* outbreak among newborns due to usage of contaminated baby shampoo [2]; another study held in Italy, reported that *Pseudomonus aeruginosa* was the most frequently isolated micro-organism from microbiologically contaminated cosmetics [3]. Product recall as a result of microbial contamination can lead to an enormous financial loss for manufacturing companies and can simultaneously affect the company's reputation [4].

The microbial examination of non-sterile topical preparations according to the United States Pharmacopeia (USP) involves the determination of

total microbial, yeast and mold counts, and testing for the absence of *Pseudomonas aeruginosa* and *Staphylococcus aureus* [5]. Conventional methods require enumeration, isolation, and identification of microbial contaminants in finished products [6]. Alternative rapid methods for bacterial identification are becoming available; PCR has led to the development of improved, convenient, and reliable methods for microbial identification and surveillance [7]. Several studies highlighted the possible role of PCR in the pharmaceutical industry [8 - 9].

MALDI-TOF is an emerging technology for microbial identification and diagnosis. In the last few years MALDI-TOF had many applications in clinical microbiology due to the speed of identification, ease of use and low cost per sample [10 - 11]; however it has few applications in pharmaceutical industry [12 - 13]. Extensive research for the possible application of MALDI-TOF in microbiological quality control in pharmaceutical industry is needed.

The aim of the present study was to investigate the potential use of MALDI-TOF for the rapid identification of *Pseudomonas aeruginosa* and *Staphylococcus aureus* in topical pharmaceutical products and to compare to PCR and conventional methods regarding time, cost-effectiveness and ease of application.

MATERIAL AND METHODS:

Bacterial Strains: The list of bacterial strains used in the study included: *Pseudomonas aeruginosa* ATCC 9027, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 6538, *Staphylococcus aureus* ATCC 25293, *Bacillus cereus* ATCC 10876, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* isolates M1 & M2 (lab collection of Department of Microbiology & Immunology, Faculty of Pharmacy, Cairo University). An optical density curve was plotted for *Pseudomonas aeruginosa* ATCC 9027 and *Staphylococcus aureus* ATCC 6538, and serial dilutions with cell counts ranging from (10° to 10^6) CFU/ml were prepared [14].

Artificial Inoculation of Finished Products and Raw Materials: Four pharmaceutical formulations: cream, ointment, gel, and emulgel; three different raw materials: Gum, maize starch, and magnesium stearate were used in the study. Serial dilutions of *Staphylococcus aureus* ATCC 6538 and *Pseudomonas aeruginosa* ATCC 9027 ranging from (10^0 - 10^6) CFU/ml were prepared in TSB in case of raw materials, or TSB containing 0.5% lecithin and 4% tween 20 in case of finished products. The finished products and raw materials were added to TSB with a dilution factor 1:10 (USP 2011) [5]. The artificially inoculated samples were overnight incubated at 35 °C.

Duplex PCR Identification of *Staphylococcus aureus* and *Pseudomonas aeruginosa* in Artificially Contaminated Finished Products and Raw Materials: Genomic DNA extraction was performed using QIAmp DNA mini kit according to manufacturer's instructions (QIAGEN, USA). The amount of DNA in the extracts was measured using Qubit® 2.0 fluorometer (Thermo Fischer Scientific, USA). Primers targeting *opr*L for *Pseudomonas aeruginosa* (oprL-F 5'-CGC GCG TGC TGA TGC TCG TAT-3'; oprL-R 5'-GCG CGA GGA ACG TCA GGA CAC-3') [7] and mRNA nuclease gene for *Staphylococcus aureus* (mRNA-F 5' TTC GAA AGG ATA CGC AAA GA 3'; mRNA-R 5' TAG CCA AGC CTT GAC GAA CTA AAG C3') [15] were synthesized by Macrogen, USA.

PCR was carried out in a 50 µl reaction volume containing Emerald Amp GT PCR master mix (Takara, Japan) using primers' concentration of 0.2µM each and 2µl Genomic DNA in Gene AMP PCR System 9700 (Applied Biosystems, USA). PCR cycling parameters were denaturation at 95° C for 5 min, followed by 35 cycles: 95°C for 30 sec., 53.3^o C for 30 sec., 72°C for 1 min, and a final extension at 72°C for 7 min. The amplified PCR products were separated by electrophoresis in 1.5% w/v agarose using 1X TAE buffer and visualized by staining with ethidium bromide. Fragment size was determined by comparison with 100 bp DNA Ladder (Sigma Aldrich, USA). The gel was examined and photographed under UV illumination[16]. PCR amplification products of oprL in *Pseudomonas aeruginosa* ATCC 9027, and *Pseudomonas aeruginosa* ATCC 27853; mRNA nuclease gene in *Staphylococcus aureus* ATCC 6538, and *Staphylococcus aureus* ATCC 25293 were purified by QIAquick PCR purification kit

according to manufacturer's instructions (QIAGEN, USA); the purified PCR products were sequenced by ABI 3730 xl DNA sequencer (Applied Biosystems, Foster City, CA). The detection of similarity searches for the nucleotide sequences was performed using BLASTN program (http://www.ncbi.nlm.nih.gov/blast).

The specificity of duplex PCR was evaluated using common pharmaceutical bacterial contaminants: *P. aeruginosa* ATCC 9027*, Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 6538*, Staphylococcus aureus* ATCC 25293*, Bacillus cereus* ATCC 10876*, Bacillus subtilis* ATCC 6633*, Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* isolates M1 & M2; the sensitivity of duplex PCR was also checked using artificially inoculated samples containing an initial bacterial count ranging from (10° - 10^6) CFU/ml, before and after the pre-enrichment step in TSB.

MALDI TOF MS Biotyper Identification of *Pseudomonas aeruginosa* and *Staphylococcus aureus* in Artificially Contaminated Finished Products and Raw Materials: The identification of microorganisms by MALDI-TOF was performed using Microflex LT/SH (Bruker Daltonics, Germany), Flex control 3.19 and MALDI Biotyper 3.1 (MALDI-TOF 3.1) (Bruker Daltonics). Three methods were tested for sample preparation using MALDI TOF MS Biotyper: the Direct Transfer method, according to manufacturer's instructions (Bruker Daltonics, Germany), Formic acid extraction method (FA) [11], and a third method, we called the direct pellet method, according to Íñigo and co-workers (2016) with some modifications [17].

In the direct pellet method, one ml of the overnight artificially inoculated broth of each sample was centrifuged at 13,000 rpm for 5 minutes, the supernatant was discarded, and the pellet was washed twice with HPLC grade water. The deposited cells were directly smeared on MALDI target plate, overlaid with one µl of alpha-cyano-4-hydroxycinnamic acid matrix (HCCA) solution and allowed to dry.

In case of emulgel samples, filtration prior to centrifugation was performed to improve the quality of recovered pellets. All samples were done in duplicates.

The MALDI-TOF analyzed samples took scores ranging from 0-3 according to the consistency of identification as proposed by the manufacturer (Bruker Daltonics, Germany). Score ≥ 2 indicated secure precise identification to genus level, an intermediate score value [1.7≤ score ≥ 1.99] indicated probable identification to genus level and a score of less than 1.7 was considered unreliable identification. Analysis of samples with a score < 2, was repeated with few modifications including an extra washing step with HPLC grade water for the removal of any residual sample from the deposited pellet, and the application of the proper amount of biological material to target plates.

The ability of MALDI TOF MS Biotyper to identify mixed cultures of *Staphylococcus aureus* and *Pseudomonas aeruginosa* was validated using two sample preparation methods: FA method and direct pellet transfer method. The specificity of MALDI TOF MS Biotyper was tested using common pharmaceutical bacterial contaminants: *P. aeruginosa* ATCC 9027*, Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 6538*, Staphylococcus aureus* ATCC 25293*, Bacillus cereus* ATCC 10876*, Bacillus subtilis* ATCC 6633*, Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* isolates M1 & M2.

RESULTS:

Identification of Pharmaceutical Contaminants in Finished Products and Raw Materials Using duplex PCR: PCR products of the expected band size of 461 bp for *Staphylococcus aureus* (mRNA nuclease) and 709 bp for *Pseudomonas aeruginosa* (*opr*L) were detected. Duplex PCR successfully detected the co-presence of both pathogens as shown in **Fig. 1**. Prior to the pre-enrichment step, no bands were observed with the artificially inoculated samples; on the other hand, both microorganisms were detected at an initial cell count of 10^0 CFU/ ml or g sample after the pre-enrichment step. No interference or cross-reactivity was observed during testing the specificity of duplex PCR to identify different strains of *Staphylococcus aureus* and *Pseudomonas aeruginosa*, or other possible pharmaceutical contaminants. The total time required for bacterial identification was reduced to 27 H and vary depending on the number of samples. The

sequenced products were deposited in the GenBank under accession numbers: MG652364-MG652367.

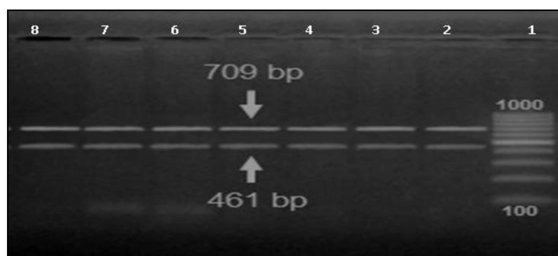

FIG. 1: AGAROSE GEL OF PCR AMPLIFICATION OF mRNA NUCLEASE FOR *STAPHYLOCOCCUS AUREUS* AND *opr*L FOR *PSEUDOMONAS AERUGINOSA* USING QIAmp DNA MINIKIT

Lane 1: 100 bp DNA Ladder ranging from 100 to 1000 bp

Lane 2-8: Artificially inoculated different pharmaceutical finished products and raw materials with mixed inoculums of both organisms: Cream, Emulgel, Ointment, Gel, Gum, Mg Stearate, Maize starch

Identification of Pharmaceutical Contaminants in Finished Products and Raw Materials Using MALDI-TOF: Seven different samples including finished preparations and raw materials were artificially inoculated with the tested micro-organisms, at cell count ranging from (10° - 10^6) CFU/ml, and tested for the presence of microorganisms using MALDI-TOF before and after the pre-enrichment step. No reliable results were obtained by MALDI-TOF prior to the pre-enrichment step. In this study, MALDI-TOF was used to identify the contaminant in 42 artificially inoculated, with a single bacterial contaminant, samples. Following the pre-enrichment step: 85.7% (36/42), 14.2% (6/42), 0% of samples scored >2, (1.7< x <1.9), and less than 1.7, respectively, as shown in **Table 1**. It was observed that the incidence of a score below 2 was the least with the FA extraction method (0.02%, 1/42), and the highest with the Direct Pellet Transfer (0.095%, 4/42).

TABLE 1: SCORE VALUES OF MALDI TOF BIOTYPER IDENTIFICATION OF *PSEUDOMONAS AERUGINOSA* ATCC 9027 AND *STAPHYLOCOCCUS AUREUS* ATCC 6538 AS SINGLE CONTAMINANTS IN ARTIFICIALLY CONTAMINATED SAMPLES USING DIFFERENT SAMPLE PREPARATION METHODS

Tested strains	Sample preparation method	Score value		
		x > 2	1.7 <x <1.9	x <1.7
Pseudomonus aeruginosa ATCC 9027	Formic acid extraction	7/7	0/7	0/7
	Direct transfer	7/7	0/7	0/7
	Direct pellet transfer	5/7	2/7*[a]	0/7
Staphylococcus aureus ATCC 6538	Formic acid extraction	6/7	1/7*[b]	0/7
	Direct transfer	6/7	1/7*[c]	0/7
	Direct pellet transfer	5/7	2/7*[d]	0/7

*[a]: Artificially inoculated samples were Gum & Mg stearate

*[b]: Artificially inoculated sample was Gel

*[c]: Artificially inoculated sample was Gum

*[d]: Artificially inoculated samples were Ointment & Maize starch

The ability of MALDI-TOF to identify mixed cultures of *Staphylococcus aureus* and *Pseudomonas aeruginosa* in artificially inoculated samples was tested using two sample preparation methods: FA extraction method, and direct pellet method. Both methods could detect only one of the two pathogens present in mixed cultures. In only two samples MALDI-TOF was able to detect both microorganisms in the same sample, though the score of the second microorganism was below 1.69 and thus considered unreliable.

The time required for the detection of pharmaceutical contaminants using MALDI-TOF including the pre-enrichment step varied according to the sample preparation method from 18 - 37 h. The only method that could detect mixed contaminants in the same sample was the direct transfer from single colonies which required an additional isolation step on the respective solid medium, thus increasing the time for detection to approximately 37 h. MALDI-TOF was able to detect different strains of *Staphylococcus aureus* and *Pseudomonas aeruginosa* and species of other possible pharmaceutical contaminants without interference with *Staphylococcus aureus* and *Pseudomonas aeruginosa* spectra.

A comparison of the time, cost, and steps involved in bacterial detection in pharmaceutical samples using conventional culture methods, PCR, and MALDI-TOF is shown in **Table 2**.

TABLE 2: COMPARISON BETWEEN CONVENTIONAL, PCR AND MALDI-TOF METHODS FOR THE DETECTION OF PHARMACEUTICAL MICROBIAL CONTAMINANTS IN TERMS OF TIME AND COST OF THE ASSAY

Tested method	Time of assay	Cost/ sample	Steps involved
Conventional method	48-72 h	0.169 USD	-Recovery on selective/ differential media -Biochemical/ Microscopical analysis
PCR	27 h	1.69 USD	-Genomic DNA extraction - DNA amplification - Gel electrophoresis
MALDI-TOF FA	19 h	0.206 USD	-FA extraction step -Sample plotting on target plate
Direct Transfer	37 h	0.206 USD	-Isolated colonies plotting on target plate
Direct pellet	18 h	0.206 USD	-Centrifugation/ washing of pellet -Sample plotting on target plate

FA: Formic acid extraction method

DISCUSSION: Microbial contamination of pharmaceutical products is listed to be among the top ten reasons for product recall from markets. By using rapid methods for detection of objectionable microorganisms, it would be much easier for companies to implement corrective actions and avoid huge financial losses besides maintaining consumers' health. Different studies have been concerned with the development, validation, and application of rapid methods in the field of pharmaceutical microbiology [7, 15, 18].

The main aim of this study was to detect *Staphylococcus aureus* and *Pseudomonas aeruginosa* in topical pharmaceutical products and raw materials using non-conventional methods including PCR and MALDI-TOF and to evaluate the time, cost-effectiveness, and ease of application of both approaches compared to conventional culture methods.

PCR has been accepted as a method for rapid detection of microbial contaminants in water, food and pharmaceutical samples [15, 19 - 20]. It can overcome many of the conventional methods drawbacks, most importantly the time consumption factor. The technique still faces few obstacles to be applied on regular bases in microbiological quality control in pharmaceutical industry. The most challenging obstacles are the availability and cost of different reagents required for various steps of PCR and the relative difficulty of application especially for beginners which might lead to contamination of DNA, although the automation of DNA extraction and PCR reaction preparation can overcome the second challenge at a higher cost [21].

In the current study, PCR could identify the tested pathogens in artificially contaminated samples with an initial bio-burden of 10^0 CFU/ml or g, using QIAmp DNA mini kit. Karanam and coworkers (2008) used the phenol extraction method for DNA extraction and this gave accepted results [7], although it was considered more tedious and inhibitory to PCR reactions in some cases [15]. Usage of mild lysis method for DNA extraction as described earlier in the study by Jimmenez and coworkers (1999) gave satisfactory results in the case of uniplex PCR, but the purified DNA yield using the QIAmp DNA mini kit gave better results [6].

In our study, the total time required for the bacterial identification was reduced to 26 h and vary depending on the number of samples, and the method for DNA extraction. These results are comparable with previous studies that stated that the time required for microbial contaminants' detection could be reduced to 27 - 30 h [6, 15]. With mixed bacterial contaminants, our duplex PCR successfully indicated the presence of both pathogens with no observed interference or cross-reactivity.

For the last few years MALDI-TOF technology has been in use for microbial identification; several studies reported the use of MALDI-TOF MS for microbial identification and strain typing in clinical isolates [22], epidemiological studies [23] detection of water and food-borne pathogens [24 - 25], detection of antibiotic resistance, [26] and detection of blood and urinary tract pathogens [17, 26]. To the best of our knowledge, this is the first study on the possible application of MALDI TOF MS Biotyper in microbiological quality control in pharmaceutical industry.

In our study, two common sample preparation methods: the direct transfer method and FA method were used for MALDI-TOF detection of *Staphylococcus aureus* and *Pseudomonas aeruginosa* in artificially contaminated samples. The ability of FA extraction method to improve the results of bacterial identification was reported in several studies; the FA extraction method improved the results of bacterial identification by 2.3% for the non-correctly identified samples by the direct transfer method [28], and increased the percent of bacterial identification to species level by 30% [29]. However, in the present study, no major differences were observed in the ability of both methods to identify the microbial contaminant to secure genus identification. Generally, application of FA extraction method for sample preparation from isolated colonies consumes more time and is usually used to improve the score of identification [28]. In our study, a third sample preparation method, the direct pellet transfer method, was tested; the incidence of "Probable genus identification" results with this method was higher than with the other two tested methods, though the microbial contaminant was correctly identified in all trials.

However, the direct Pellet transfer method is considered the fastest, easiest and most cost-effective one. The time required for bacterial identification was reduced with MALDI-TOF Biotyper to 18 h for the direct pellet transfer method, 19 h for FA extraction method and 37 h for direct colony transfer method.

In case of mixed bacterial cultures, FA and direct pellet transfer methods detected only one of the two present pathogens. It is worth mentioning that the detection of one pathogen is sufficient to reject the tested sample as per USP. The only method that could detect mixed contaminants is the direct transfer from single colonies, yet this requires additional colony isolation on the respective solid medium, thus increasing the time for detection to approximately 37 h. By improving sample preparation method and the detection algorithms by the manufacturer, the sensitivity of identification of mixed cultures could be improved [11, 28, 30].

In this study, MALDI-TOF MS Biotyper was able to identify other possible pharmaceutical contaminants based on the characteristic peaks of MALDI spectra. The unique fingerprint of different strains provided a unique profile for differentiation based on the *m/z* value. It should be noted that the database of MALDI-TOF should be continuously improved and updated, this will in return help to increase its specificity and ability for microbial detection.

Our results showed that the pre-enrichment step was essential for the detection of microorganisms in both PCR and MALDI-TOF MS Biotyper. This may be attributed to the low level of the initial microbial count. The pre-enrichment step allows the multiplication of the number of indicator pathogens and thus increasing reaction sensitivity. The importance of enrichment for PCR was in accordance with previous recommendations [31].

The microbial detection limit in case of MALDI TOF MS varies from one microorganism to another and differs according to the nature of tested sample; secure MALDI TOF MS species identification scores for *Pseudomonas aeruginosa* in urine samples were observed with a bacterial count of 5×10^5 CFU/ml [28], while secure species identification scores for *Staphylococcus aureus*, directly from milk samples, were detected with a minimal bacterial count $\geq10^6$ CFU/ml [32].

In our study, lower scores of identification were recorded with *S. aureus* rather than *Pseudomonas aeruginosa* with the different tested sample preparation methods, as recorded in **Table 1**. Further studies should be conducted to test the effect of the nature of the pharmaceutical product and the microbial load, on the precise identification by MALDI TOF MS.

In our study, comparable results were obtained with MALDI-TOF and PCR; however MALDI-TOF showed some advantages over PCR in terms of the lower cost per sample, despite the high initial cost of the device that should be considered, the relative ease of implementation, the high throughput, the "all in one device" process and the high reproducibility of the process by performing the run-to-run Bacterial Test Standard (BTS) quality check before each run.

CONCLUSION: Optimization of MALDI-TOF to be used on an everyday basis can serve in establishing a solid ground for it to be applied on larger pace in pharmaceutical companies and drug research centers. This will consequently result in time, cost and effort saving and cope with the pharmaceutical market's high demands. In addition, further research should be done to improve its ability to detect mixed bacterial contaminants by improvement of the sample preparation method and device detection algorithms

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