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Quality of Non-Sterile Herbal Pharmaceutical Products – Detection of Fungi

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Abstract

The aim of this paper was to use real-time quantitative PCR (qPCR) to perform microbiological purity tests, aiming to identify the presence/absence of fungal DNA and its quantification from non-sterile pharmaceuticals, with different manufacturers and various compositions. For identification a potential presence of fungi in non-sterile drug samples, fungal DNA has been isolated, amplified and quantified under real-time qPCR conditions. qPCR analysis of non-sterile pharmaceutical samples showed the presence of fungi in all investigated non-sterile herbal drugs. The highest amount of fungal DNA was recorded for S2 sample (0.0767 ng) and the lowest fungal DNA quantities have been registered for S1 sample (0.000032 ng). qPCR-based methods provided an earlier and more sensitive detection, identification, and quantification of fungal contamination compared to standard methods.

Keywords: identification of fungi; non-sterile herbal pharmaceutical products; quality control; qPCR.

1. Introduction

Microbiological quality control of non-sterile herbal pharmaceutical products is a very important step, that has a direct impact on their efficacy and safety, and must corresponding to the requirements of the pharmacopoeia [1,2,3,4,5].

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Quality control of non-sterile herbal pharmaceutical products define the quality of the herbal substance and herbal medicinal product [6]. But, in order to achieve the quality objective, it is necessary to control all phases of pharmaceutical products, aspects that individually or collectively influence the quality of a product, including the manufacturing process and evaluation of the finished product [7,8]. Principal sources of microbial contamination of the non-sterile pharmaceutical products with bacteria, yeast, fungi and molds are raw materials, environment and manufacturing process [9,10]. Contamination of pharmaceutical products with fungi may lead to reduce or eliminate the therapeutic properties of drugs, can change physicochemical characteristics of drugs and may cause potentially infections due to the presence of toxic molecules (aflatoxins – *Aspergillus* sp.) [7,9,11]. The pharmaceutical products are susceptible to microbial contamination, especially in warm (30^oC- 31^oC) and humid conditions (75%) [4,11]. The presence of objectionable microorganisms in non-sterile pharmaceutical products indicates lack of process and quality control. Microbiological control of pharmaceuticals is important to identification of critical points of the production process and to improve the quality of production and to improve the safety and quality of drugs [7]. There are several reasons why a drug can be recall from the market, but fungal contamination of non-sterile pharmaceutical preparations is the most common. From 112 recalls by FDA, 23% of samples was contaminated by mold, fungi and yeast (e.g., *Aspergillus* and *Penicillium*) [12]. Some herbal non-sterile pharmaceutical products are not properly monitored and others have not been tested [13]. The future of pharmaceutical manufacturing should be six sigma quality (no more than 3.4 defects occur per million opportunities), comparatively with the current two or three sigma quality [14]. On the consumer side, eliminating drug recalls provides less risk to the consumer. Also, six sigma quality can help assure a rapid implementation of corrective actions which are particularly beneficial for minimizing manufacturing losses [14]. In accordance with GMP requirements (Good Manufacturing Practices), manufacturers must to apply microbial contamination control practices during the production process [15,16,17]. Both European Pharmacopoeia (EP) and United States Pharmacopoeia (USP) contain protocols to determine the microbiological quality of non-sterile products that comprise either quantitative methods for counting microorganisms or qualitative tests to show the presence/absence of specified organisms [15,16,17]. Conventional microbiological methods are laborious, time-consuming, lack sensitivity and, require several days of incubation. Furthermore, another limitation of the methods is the inability to determine the presence of the viable but non-culturable (VNC) microorganisms, which are uncultivable by classical methods [18,19,20,21,22]. Rapid microbiological methods (RMMs) are more sensitive, accurate, precise and automated and provide data on quantity and quality of microbes present in the sample. RMMs are described as alternative methods in some relevant chapter of the guidance [2,3,23,24,25] and, involve technologies that can be growth-based, viability-based, or surrogate-based cellular markers for a microorganism (e.g., nucleic acid based) [19,24,25]. DNA/RNA-based methods that offer real-time results (polymerase chain reaction - PCR and qPCR) are rapid methods for sensitive and DNA quantifying can be used for detection of bacteria and fungi [11,24,25]. The aim of this paper was to use qPCR to perform microbiological purity tests, aiming to identify the presence/absence of fungal DNA and its quantification from non-sterile pharmaceuticals, with different manufacturers and various compositions.

2. Materials and methods

Five samples of non-sterile herbal pharmaceutical products, manufactured by different pharmaceutical factories

were introduced into this study. Samples, in the form of tablets and capsules, with the oral route of administration were labelled S1-S5. The samples were opened only at the time of analysis, and the experiments were performed under sterile conditions. Fungal DNA isolation was performed with Quick-DNA Fungal/Bacterial Microprep kit (Zymo Research, USA) using 50 mg of each sample. DNA isolation procedure was performed according to the producer recommendation. The lysis procedure was fast and efficient. For the purification of the isolated DNA, Zymo-Spin™ technology was used. The elution step allows obtaining of 10 µL high quality DNA. For detection and quantification fungal DNA from samples, Femto™ Fungal DNA Quantification kit (Zymo Research, USA) it was used. Femto™ Fungal DNA Quantification kit is a real-time PCR kit and contains a primer mix, targeting the ITS region (internal transcribed spacer), negative control (NTC) and seven fungal DNA standards (20-0.00002 ng fungal DNA input/reaction well) that were purified from *Saccharomyces cerevisiae* strain TMY18. Femto™ Fungal qPCR premix includes SYTO®9 fluorescent dye. qPCR was performed in a 20 µL volume containing aliquoting Femto™ Fungal qPCR premix and unknown test samples/fungal DNA standards/no template control for each sample. Real-time qPCR analysis was carried out in an IQ^5 thermocycler (Bio-Rad). To achieve the repeatability of the method, all samples (five unknown samples S1-S5, seven fungal DNA samples and negative control) were prepared in duplicate. The real-time qPCR amplification conditions were: initial denaturation 10 min. at 95^{o}C, 45 cycles of denaturation 30 sec. at 95^{o}C, annealing 30 sec. at 50^{o}C, extension 1 minute at 72^{o}C and final extension 7 minutes at 72^{o}C.

3. Results

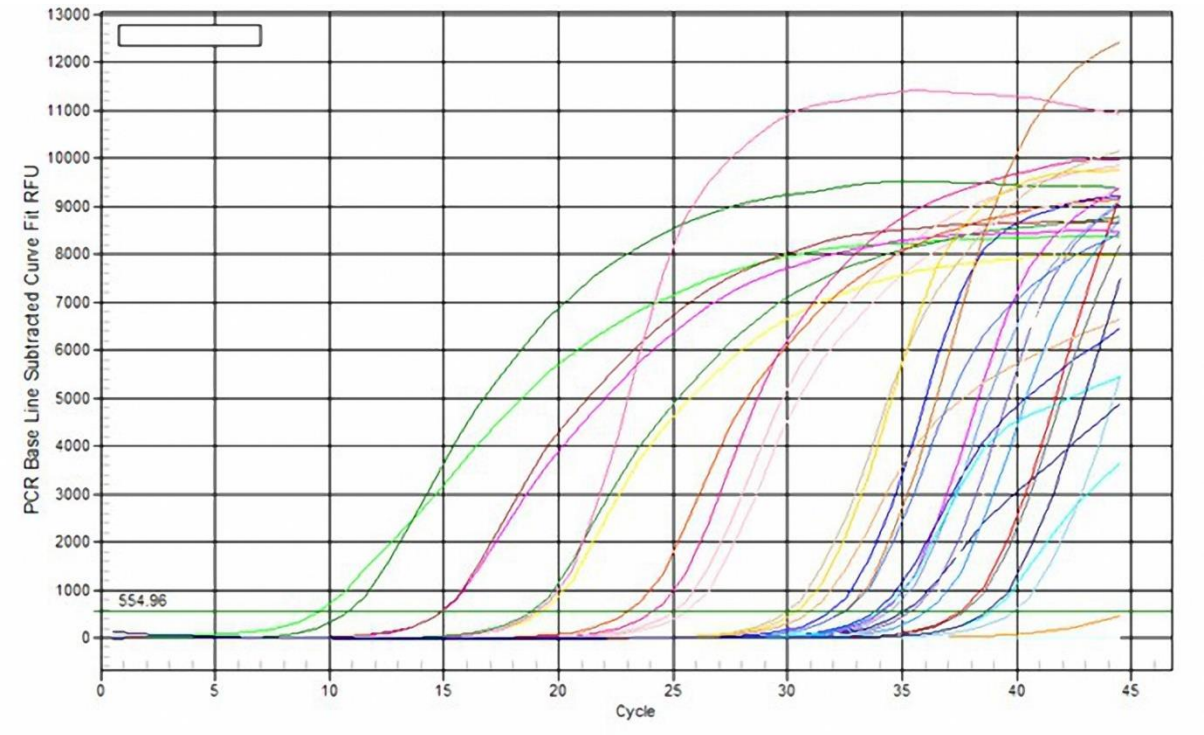

Figure 1: Amplification chart of five unknown samples (S1-S5), seven fungal DNA standards and negative control (NTC)

qPCR analysis of five analyzed samples showed the presence of fungal DNA in all investigated non-sterile

samples. In the amplification chart (Figure 1) it is observed that five unknown samples analyzed in comparison with the standards had positive amplification signals. The quantification strategy used in the amplification reaction was absolute quantification using calibration curve and measuring Ct (quantification cycles - Cq values, Ct-cycle threshold) to determine starting copy number. The R^2 value recorded in the calibration curve and drew for the standard samples and for unknown samples, was 0.993, which shows that qPCR amplifications were effective (Figure 2).

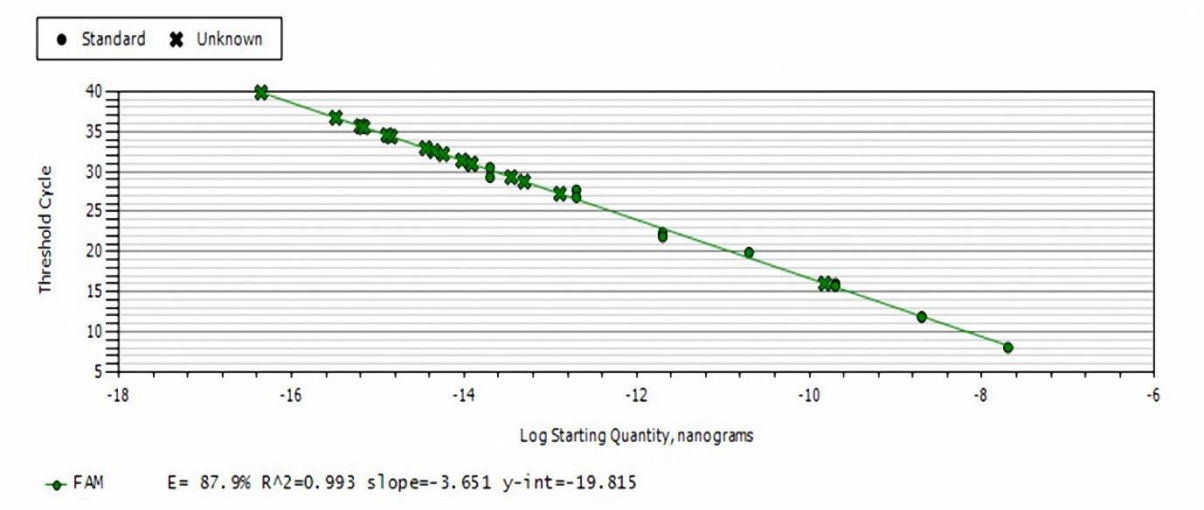

Figure 2: PCR standard curve

For S2 sample (with low Cq), the amount of fungal DNA recorded was higher: 7.67 x 10^{-11}, which represents 0.0767 ng, values close to that recorded for the STD4 sample. The lowest fungal DNA quantities have been registered for S1 sample (3.2 x 10^{-14} – 0.000032 ng), similar with amount of fungal DNA recorded for STD7 sample. In case of the negative control samples (NTC), the quantity of the amplified product was 0, which shows that the analyzed samples were not contaminated during processing (Figure 1). After the quality control performed for five pharmaceutical products by molecular methods, it was found that the Cq values represents strongly positive reactions, with an abundant amount of target sequence in the sample and positive reactions with a moderate amount of target sequence in the sample. The higher load of fungal DNA in the S2 sample could be due to the presence in the product, material from four different species. qPCR analysis is a complex method that involves expensive equipment and special procedures, but qPCR method is accurate and precise.

4. Discussion

Generally, fungal contamination of products may be due to the raw materials used, the storage conditions of the products or the packaging materials used [25]. Based on scientific studies has identified numerous cases of contamination of non-sterile products with Gram-negative bacteria, Gram-positive bacteria and fungi too [7,9,11,12,13,17]. Using traditional methods, an analysis of non-sterile drugs from Poland indicated unconformities regarding microbial loading and high levels of the number of fungi ($3.4x10^3$ CFU/g) (*Aspergillus* spp., *Rhizopus* spp., *Alternaria* spp., *Mucor* spp.) [7]. Another study about microbiological purity of dietary supplements containing plant-based ingredients showed that the samples were contaminated by a high

number of aerobic bacteria and fungi (7.0 x10^4 CFU/g). The samples showed contamination with different species of fungi: *Aspergillus* spp., *Alternaria* spp., *Penicillium* spp., *Fusarium* spp., *Rhizopus* spp., *Mucor* spp. [26]. Comparatively with conventional methods, in this study, qPCR analysis identity the presence of fungal DNA and rapid detect low levels of fungal contamination in pharmaceutical samples, but does not provide data on the type of fungal species. Due to the negative effects, they can have on pharmaceuticals, it is important to quickly detect them in different types of pharmaceutical products. Evaluation of some medicinal herbs emphasized that samples were contaminated with fungi that had a very high toxigenic capacity (*Aspergillus* spp., *Fusarium* spp.) [27,28]. The authors in [9] have evaluated fungal contaminants in some ointments and tablets after opening of the packages in hospitals and have reported that some of the ointments and tablets were contaminated by *Aspergillus fumigatus* and *Aspergillus flavus* before and after opening the coverage. Some fungi produce toxic metabolites and may cause rapid deterioration of the product. The authors in [29] have described novel cases of microbial contamination of non-sterile pharmaceuticals in public hospital settings. The study revealed fungal contamination, including *Aspergillus* spp. and *Candida albicans*. Their presence may cause a deterioration of the patients` health. Another study about quality herbal products sold in Thailand showed that 11% of examined products were contaminated with yeasts/molds [30]. Other studies have shown the importance of rapid and sensitive detection techniques (RMMs), particularly PCR techniques, for microbiological control of pharmaceutical products. PCR-based methods are faster and more sensitive, compared with standard methods, regarding detection and identification of bacterial and fungi contamination [11,17,21,22,24,31]. At the same time, authenticity of all ingredients in herbal pharmaceutical preparations, is a fundamental requirement to establish the purity and the quality of the product [32], using next-generation sequencing [33]. Because most herbal products are poorly monitored and fungal can be present in environments with low humidity levels, the results of this study demonstrate that qPCR analysis could be used for faster and sensitive monitoring of pharmaceuticals and raw materials and can provide information on the quality of the analyzed product. Using rapid methods can improve the quality of herbal pharmaceutical production, can inform about the need for microbiological control in the production process of the types of drugs tested and therefore can improve the safety and quality of drugs.

5. Conclusions

The results obtained in this study illustrate the importance of the qPCR method for the microbiological control of non-sterile pharmaceuticals, the method having the ability to detect, identify and quantify very small amounts of fungal DNA in samples.

6. Conflict of interest

The author declare no conflict of interest to this work.

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