

## DETECTION OF *ESCHERICHIA COLI* FROM NON-STERILE HERBAL PHARMACEUTICAL PRODUCTS FROM THE ROMANIAN MARKET

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### Abstract

The purpose of the study was to demonstrate the importance of using rapid microbiological methods, particularly real-time quantitative polymerase chain reaction (qPCR), for the rapid detection of *Escherichia coli* from non-sterile herbal pharmaceutical products available on the Romanian market. For the identification of the potential presence of *Escherichia coli* in 10 non-sterile drug samples, genomic bacterial DNA has been isolated, amplified and quantified under real-time qPCR conditions. qPCR analysis of 10 analysed samples showed the presence of *Escherichia coli* in all investigated non-sterile herbal drugs. The highest amount of bacterial DNA was recorded for 2 samples (3.58 ng and 1.66 ng) and the lowest bacterial DNA quantities have been registered for the sample S10 (0.00013 ng). PCR-based methods provided a rapid detection and quantification of bacterial contamination that showed the presence of *Escherichia coli* in all investigated non-sterile herbal pharmaceutical products.

### Rezumat

Scopul acestui studiu a fost de a arăta importanța utilizării metodelor microbiologice rapide, în special a PCR cantitativ în timp real (qPCR) pentru detecția *Escherichia coli* din produse farmaceutice nesterile, existente pe piața din România. Pentru identificarea prezenței *Escherichia coli* în 10 probe farmaceutice nesterile, ADN-ul bacterian genomic a fost izolat, amplificat și cuantificat prin metoda qPCR. Analizele qPCR ale celor 10 probe au arătat prezența *Escherichia coli* în toate probele investigate. Cele mai mari cantități de ADN bacterian au fost înregistrate pentru 2 probe (3,58 ng și 1,66 ng) și cea mai mică cantitate de ADN bacterian a fost înregistrată pentru proba S10 (0,00013 ng). Metodele bazate pe qPCR oferă o detecție și o cuantificare rapidă și sensibilă a contaminării cu *Escherichia coli*.

**Keywords:** *Escherichia coli*, non-sterile herbal pharmaceutical products, real-time quantitative PCR (qPCR)

### Introduction

The microbiological quality control of the pharmaceutical products is a very important topic to reduce the risk of the user [9]. Non-sterile pharmaceutical products are not produced by aseptic processes [27]. Non-sterile pharmaceutical products can be contaminated with bacteria, yeast, fungi and molds. The microbial contamination may lead to reduce or eliminate the therapeutic properties of drugs, change the contents of active ingredients and may cause potentially infections due to the presence of the pathogenic microorganisms that may produce toxic metabolites [15, 19, 27]. The presence of the microorganism toxins may cause some diseases such as diarrhoea, acute gastroenteritis, and abdominal pain [15, 19]. Even dry pharmaceutical products are susceptible to microbial contamination, especially in warm and humid climates [27]. Most Gram-positive bacteria, such as *Enterococcus* spp., *Staphylococcus aureus* or *Streptococcus pyogenes*, and many Gram-negative species, such as *Acinetobacter* spp., *Escherichia coli*, *Klebsiella* spp., *Pseudomonas aeruginosa*, *Serratia marcescens* or *Shigella* spp. survive for months on dry surfaces [13, 28].

GMPs (Good Manufacturing Practices) require the manufacturers to apply microbial contamination control practices during the production process and to develop microbial specifications for non-sterile products [9]. The degree of contamination in non-sterile products and the acceptance criteria according to the route of administration, it was regulated in the European Pharmacopoeia (Ph. Eur.) and the United States Pharmacopoeia (USP) [19, 27, 28]. Ph. Eur. and USP limits test require the absence the following undesirable microorganisms: *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella typhimurium* and *Candida albicans* [10, 27, 28]. Both Ph. Eur. and 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 the specified organisms [3, 22, 23].

Traditional microbiological methods using conventional growth-based methods are time-consuming, labour intensive and they lack sensitivity, and require several days of incubation [5, 15, 22, 23]. A further limitation of the traditional microbiological methods is the cultivability and the presence of the viable, but non-

culturable (VNC) microorganisms. Many different types of bacteria, although remaining physiologically active, are viable but non-culturable (VNC). VNC organisms are capable of product spoilage and may be a potential infectious threat. [5, 15, 16, 18].

Comparatively with conventional methods, rapid microbiological methods (RMMs) are usually automated, more sensitive, accurate, precise and reproducible tests. RMMs involve technologies that can be growth-based, viability-based, or surrogate-based cellular markers for a microorganism (e.g., nucleic acid based, fatty acid based) [5, 15, 18]. In addition, direct fluorescent labelling techniques offer a suitable approach to the detection of the VNC organisms [5]. RMMs are described as alternative methods in some relevant chapter of the guidance, where they were defined validation criteria for RMMs: Ph. Eur. 5.1.6. "Alternative Methods for Control of Microbiological Quality" (7<sup>th</sup> edition, 2011), Ph. Eur. 2.6.27 "Microbiological Control of Cellular Products" (7<sup>th</sup> edition, 2011), USP <1223> "Validation of Alternative Microbiological Methods" (34<sup>th</sup> edition, 2011) [21].

The purpose of the study was to underline the importance of using rapid microbiological methods, particularly real-time quantitative PCR (qPCR), to control the quality of non-sterile herbal pharmaceutical products. In this regard, qPCR-based assay was used for rapid detection of *Escherichia coli* from non-sterile herbal pharmaceutical products placed on the Romanian market.

### Materials and Methods

Ten samples of non-sterile drugs from different Romanian markets and manufactured by different pharmaceutical factories were introduced into this study. Samples, in the form of tablets, capsules, chewable tablets having the oral route of administration were labelled S1-S10. The samples were opened only at the time of analysis, and the experiments were performed under sterile conditions.

Bacterial 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<sup>TM</sup> technology was used. The elution step allows to obtain 10 µL high quality DNA.

For the identification of the potential presence of *Escherichia coli* in ten DNA samples of non-sterile drug and for genomic DNA quantification, Femto<sup>TM</sup> Bacterial DNA Quantification kit (Zymo Research, USA) it was used. Femto<sup>TM</sup> Bacterial DNA Quantification kit is a real-time PCR kit and contains a primer mix, targeting the 16S rRNA, negative control (NTC) and seven bacterial DNA standards (20 - 0.00002 ng bacterial DNA input/reaction well) that were purified

from *E. coli* strain JM109. Femto<sup>TM</sup> Bacterial qPCR premix includes SYTO<sup>®9</sup> fluorescent dye.

qPCR was performed in a 20 µL volume containing aliquoting Femto<sup>TM</sup> Bacterial qPCR premix and analysed test samples/bacterial DNA standards/no template control for each sample. Real-time qPCR analysis was carried out in an IQ<sup>5</sup> thermocycler (Bio-Rad). All samples (ten analysed samples S1-S10, seven bacterial DNA standards samples and negative control) were prepared in duplicate. The real-time qPCR amplification conditions were: initial denaturation 10 minutes at 95°C, 40 cycles of denaturation 30 sec. at 95°C, annealing 30 sec. at 50°C, extension 1 minute at 72°C and final extension 7 minutes at 72°C.

Data analysis was performed using GraphPad InStat 3.05 demo version (2000) software.

### Results and Discussion

Taking into account the provisions regarding the limits allowed for microorganisms in the non-sterile pharmaceutical products (the absence of *Escherichia coli*), the ten samples were tested by molecular techniques, using qPCR method.

qPCR analysis of ten analysed samples showed the presence of *Escherichia coli* in all investigated non-sterile drugs. In the amplification chart (Figure 1) it is observed that the ten investigated samples analysed in comparison with the standards had positive amplification signals.

The quantification strategy used in the amplification reaction was absolute quantification, using calibration curve. The R<sup>2</sup> values recorded in the calibration curve and drew for the standard samples and for analysed samples, were higher than 0.95 (0.993), which shows that qPCR amplifications were effective.

The software of the thermal cycle automatically records the quantification cycles – Cq values (Ct-cycle threshold), which represents the number of cycles required to produce a constant emission of fluorescence (Figure 1). The fluorescence is recorded when amplification curve crosses with the values of the fluorescence threshold signal for each sample.

In case of the negative control samples (NTC), the quantity of the amplified product was 0, which shows that the analysed samples were not contaminated during processing (Figure 1).

Table I shows the mean values and standard deviations recorded for both Cq (Ct) and quantity of bacterial DNA, in all the investigated samples.

For S7 and S8 samples (with low Cq), the amount of bacterial DNA recorded was higher:  $3.58 \times 10^{-9}$ , which represents 3.58 ng and  $1.66 \times 10^{-9}$ , which represents 1.66 ng, values close to that recorded for the STD 2 sample ( $2 \times 10^{-9}$  - 2 ng). The lowest bacterial DNA quantities have been registered for the S10 sample ( $1.30 \times 10^{-13}$  - 0.00013 ng), similar with the amount of

the bacterial DNA recorded for STD6 sample ( $2 \times 10^{-13}$ ) (Table I).

After the microbiological control performed for ten pharmaceutical products by molecular methods, it was found that the Cq values were lower than 29, which

represents strongly positive reactions, with an abundant amount of target sequence in the sample. A PCR efficiency corresponds to a slope of -2.691 that means a good amplification reaction [2].

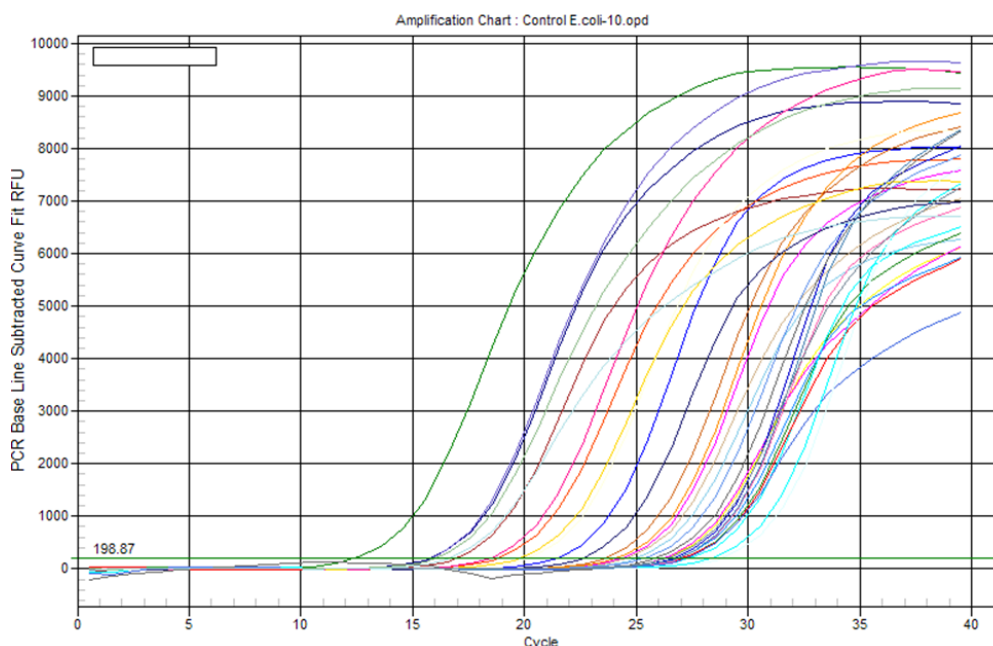


Figure 1.

Amplification chart of ten analysed samples (S1 - S10), seven bacterial DNA standards and negative control (NTC)

Table I

Representation of average data for all investigated samples

| Sample | Ct (Cq) mean | Ct (Cq) standard deviation | Bacterial DNA quantity mean     | Bacterial DNA quantity standard deviation |
|--------|--------------|----------------------------|---------------------------------|---|
| Std1   | 12.35        | 0.386                      | 2.00E-8 $2 \times 10^{-8}$      | 0.00E + 00                                |
| Std2   | 15.87        | 0.104                      | 2.00E-9 $2 \times 10^{-9}$      | 0.00E + 00                                |
| Std3   | 18.71        | 0.205                      | 2.00E-10 $2 \times 10^{-10}$    | 0.00E + 00                                |
| Std4   | 21.99        | 0.079                      | 2.00E-11 $2 \times 10^{-11}$    | 0.00E + 00                                |
| Std5   | 24.09        | 1.579                      | 2.00E-12 $2 \times 10^{-12}$    | 0.00E + 00                                |
| Std6   | 27.02        | 0.403                      | 2.00E-13 $2 \times 10^{-13}$    | 0.00E + 00                                |
| Std7   | 28.70        | 0.221                      | 2.00E-14 $2 \times 10^{-14}$    | 0.00E + 00                                |
| S1     | 24.77        | 0.367                      | 1.00E-12 $1.00 \times 10^{-12}$ | 3.10E-13                                  |
| S2     | 26.26        | 1.005                      | 3.26E-13 $3.26 \times 10^{-13}$ | 2.50E-13                                  |
| S3     | 26.66        | 0.498                      | 2.04E-13 $2.04 \times 10^{-13}$ | 8.42E-14                                  |
| S4     | 23.93        | 0.327                      | 2.05E-12 $2.05 \times 10^{-12}$ | 5.66E-13                                  |
| S5     | 20.08        | 0.290                      | 5.53E-11 $5.53 \times 10^{-11}$ | 1.36E-11                                  |
| S6     | 27.01        | 0.811                      | 6.01E-13 $6.01 \times 10^{-13}$ | 3.74E-13                                  |
| S7     | 16.37        | 0.436                      | 3.58E-09 $3.58 \times 10^{-9}$  | 2.27E-09                                  |
| S8     | 19.28        | 3.225                      | 1.66E-09 $1.66 \times 10^{-9}$  | 3.13E-10                                  |
| S9     | 24.28        | 2.334                      | 3.26E-12 $3.26 \times 10^{-12}$ | 4.09E-12                                  |
| S10    | 27.14        | 0.025                      | 1.30E-13 $1.30 \times 10^{-13}$ | 2.82E-15                                  |

Std1-Std7 = bacterial DNA standards, NTC = negative control, S1 - S10 = investigated samples

For statistical analysis, the duplicate values representing bacterial DNA quantities from ten analysed samples (S1 - S10) were compared using the t-test. Correlation coefficient (r) was 0.8368 and p value was 0.0025, considered highly significant.

Non-sterile pharmaceutical products must conform to the microbiological purity criteria set out in guidance

(Ph. Eur.) – absence of *Escherichia coli* (1 g, 1 mL) [19, 30]. Control of the herbal products is a preventive mechanism [19]. So, the presence of *Escherichia coli* in non-sterile products indicates the lack of rigorous process control (raw materials, manufacturing process and finished product) [19, 30].

Based on the scientific studies, *Escherichia coli* is one of the microbial contaminants in non-sterile pharmaceutical products [1, 10, 19], besides a few other cases of infection with: *Enterococcus* genus [19], *Bacillus* spp. [14, 30], *Staphylococcus aureus* [1, 8, 10, 20], *Pseudomonas aeruginosa* [10], *Salmonella* spp. [1, 11], *Shigella* spp. [9], *Klebsiella* spp. [14], *Candida* spp. [14].

Modern research has identified numerous cases of contamination of non-sterile products, both with Gram-negative bacteria and Gram-positive bacteria too [4, 19, 30]. Some of these species should be considered as undesirable in pharmaceuticals [4].

An analysis of non-sterile drugs from Poland indicated that 24 samples showed non-conformities regarding microbial loading. The aerobic bacteria that led to the contamination of the tested products belong to the following microorganisms: *Bacillus*, *Micrococcus* and *Enterococcus*. Also, three samples contained *Enterobacteriaceae* bacteria, and two samples (oral dosage forms) were infected with *Escherichia coli*. The source of contaminants may be in the natural environment (water and soil) [19].

Another study about contamination of herbal medicinal products marketed in Kaduna Metropolis showed that 58.7% of the samples were contaminated by *Escherichia coli*, which is an indicator for faecal contamination, 46.7% were contaminated by *Salmonella typhi* and 65.7% by *Staphylococcus aureus*, while 19.3% were contaminated by *Shigella* spp. [1].

The microbial evaluation of some pharmaceutical non-sterile preparations used in the Egyptian market emphasized that some preparations were contaminated by *Staphylococcus* species. Observed contaminated could suggest contamination from the equipment and/or raw materials [8].

Osungunna *et al.* have evaluated the microbial quality of selected blister-packed paracetamol tablets and paracetamol syrups marketed in Nigeria and have reported that ten syrups were contaminated with: *Escherichia coli*, *Klebsiella* spp., *Pseudomonas aeruginosa* and *Staphylococcus aureus* [17].

Other studies have shown the importance of rapid and sensitive detection techniques (RMMs), particularly by PCR techniques, for microbiological control of pharmaceutical products [11, 20, 26]. Thus, a PCR-based assay has been used for *Salmonella* spp. detection in raw materials and cosmetic/pharmaceutical products [11], for *Staphylococcus aureus* detection in pharmaceutical preparations [20] and for rapid screening of *Staphylococcus aureus* and *Pseudomonas aeruginosa* in pharmaceutical products for topical use [26]. Farajnia *et al.* have developed a multiplex polymerase chain reaction assay for simultaneous detection and identification of four indicator pathogenic bacteria in a single reaction, namely: *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Salmonella* spp. [7]. In this study, it was shown a simultaneous detection of low levels of

four major potential pathogenic bacteria, in pharmaceutical finished products using a multiplex PCR reaction in a cost-effective and timely manner [7].

Several reports have been published describing the clinical dangers posed by microbiological contaminated pharmaceuticals [19] and have resulted in products recall [14]. Most of recalls were attributed to the presence of “objectionable”/undesirable organisms like *Burkholderia (Pseudomonas) cepacia* [12]. Presence of *Burkholderia cepacia* in non-sterile products may enter the nasal passage or the lungs, especially in the case of susceptible populations to pneumonia [12]. Jimenez *et al.* have developed a real-time polymerase chain reaction (RT-PCR) to detect *Burkholderia cepacia* in pharmaceutical products, allowing by this way faster quality control analysis, corrective actions, and process optimization [12].

Stickel *et al.* have described two novel cases of severe hepatic injury after intake plant products contaminated with *Bacillus subtilis* [25]. For microbiological analysis, *Bacillus subtilis* was identified by sequencing the 16S rRNA and *gyrB* genes, and analysis of the cellular fatty acids [25]. Moreover, several cases have been reported with severe liver injury after the intake of several plant products [6, 24, 25].

To improve the safety and quality of the pharmaceutical products, a higher level of hygiene must be insured during manufacturing [30] and the correctness of the current specifications, both for the raw materials and for the finished products [19].

Yu and Kopcha consider that the future of pharmaceutical manufacturing should be six sigma quality (no more than 3.4 defects occur per million opportunities) for both consumer healthcare and economic reasons, comparatively with the current two or three sigma quality [29]. 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 and consistent performance, which are particularly beneficial for minimizing manufacturing losses [29].

## Conclusions

qPCR analysis is a sensitive technique that detects low amount of bacterial DNA. PCR-based methods provided a rapid detection and quantification of bacterial contamination that showed the presence of *Escherichia coli* in all investigated non-sterile herbal pharmaceutical products.

## Conflict of interest

The authors declare no conflict of interest.

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