

Review on Application of Nucleic Acid Amplification Techniques in Pharmaceutical Products Analysis

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ARTICLE INFO

Article history:

Received on: 19/10/2015

Revised on: 07/11/2015

Accepted on: 20/11/2015

Available online: 27/12/2015

Key words:

Nucleic acid amplification techniques, Polymerase chain reaction, Pharmaceutical products, rapid methods, ATP.

ABSTRACT

The aim of this study was to describe application of molecular diagnostic tests based on nucleic acid amplification technologies (NAATs) in pharmaceutical product analysis. NAAT have become widely established in clinical microbiology laboratories in recent years, as well as in quality control (QC) laboratories for food testing, and lately introduced to the pharmaceutical QC laboratories. The number of available nucleic acid and gene amplification-based rapid microbiological methods has increased over the last few years, and for good reason. When compare to standard culture based methods, NAAT provide a rapid, an accurate and reliable means for detecting specific microorganisms of interest, especially in pharmaceutical dosage forms that are required to be free of objectionable or specified pharmacopeial organisms. This article discussed about the different types of nucleic acid amplification techniques and methods available to pharmaceutical microbiologists working on quality control of pharmaceutical products and develop some awareness among other pharmaceutical scientists. This review also highlights limitations of these methods applied in industrial setup.

INTRODUCTION

Deoxyribonucleic acid (DNA) contains the genetic information that controls the development of a microbial cell. DNA determines the genotypic and phenotypic potential of a microbial cell. With the latest advances in genomics, where more than 25 microbial genomes have been sequenced, the potential to use genetic information for the detection and discrimination of microorganisms is endless. Genetic technologies can increase the resolution and specificity of microbial detection and identification in pharmaceutical environments. DNA-based technologies are used in clinical, food, and environmental samples providing valuable information on the survival, distribution and function of microorganisms in those habitats (Hill, 1996; Ieven and Goosens, 1997). One of the technologies based on DNA analysis is the polymerase chain reaction (PCR). It was the first nucleic acid amplification method (NAAT) developed and until now has been the method of choice since its

invention by Mullis (1990). PCR amplifies specific DNA sequences along the microbial genome. For example, a set of DNA primers is used to target the specific sequence to be amplified. The PCR reaction take place in three different steps such as denaturation, annealing and extension. The three steps are repeated again for a given number of cycles, e.g., 30–35. As soon as the target is amplified, the products are detected by gel electrophoresis. However, new systems have been developed that rely on fluorescence detection of amplified products. PCR based assays are used routinely in the food industry and clinical laboratories to detect and identify bacteria, yeast, and mold (Hill, 1996; Ieven and Goosens, 1997). Now a days, rapid methods are used to detect microbial contamination causing organisms in pharmaceutical products. Microbial Contamination in pharmaceutical products due to the presence of various microorganisms such as (*Salmonella* spp, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*) in raw materials and water, or from poor practices during product manufacturing. The presence of microorganisms in pharmaceutical products represents a serious health threat to consumer's worldwide (Underwood, 1998). Furthermore, microbial growth has a negative impact on product integrity (Sutton, 1997).

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On the basis of published scientific studies, the Enterobacteriaceae, *Pseudomonas* spp., *Burkholderia cepacia* and mould have been found to be the most common microbial contaminants around the world (Oie and Kamiya, 1996; Zani *et al.*, 1997; Jimenez, 2007). Identification of microbial contaminants provides important information to track contamination sources, implement proper corrective actions and understand microbial community composition (Jimenez, 2004; Cundell, 2006). For this purpose, there are a number of standard methods for identifying and detecting those indicator pathogens in pharmaceutical industry for quality evaluation of raw materials and finished products in different pharmacopoeias. The detection of microbial contaminants has been traditionally performed using cultivation-based methods. However, all these traditional methods of identification based on microbiological culture methods, morphological and biochemical characterization of microorganisms (Baird, 1998).

Therefore, there is a need in pharmaceutical industry to develop and perform a rapid procedure to detect the contaminants in timely manner to take immediate corrective action for contamination control and avoid huge production loss and product integrity. Molecular methodologies such as Adenosine triphosphate (ATP) bioluminescence and polymerase chain reaction (PCR)-based assays provide rapid quality control analysis of cosmetic and pharmaceutical finished products and raw materials. The new molecular methods are available that can rapidly detect microorganisms in contaminated samples.

Hence, in this review to analyze the various nucleic acid techniques available in pharmaceutical product analysis. In addition to that, this review discuss the advantages and issues of implementation of these methods in industrial setup.

VARIOUS MOLECULAR METHODS IN PHARMACEUTICAL PRODUCT ANALYSIS

During the last five years, several peer review studies have been published on the research, development, validation and application of rapid methods to pharmaceutical microbiology (Samadi *et al.*, 2007; Karanam *et al.*, 2008; Ragheb *et al.*, 2012). ATP bioluminescence, direct viable counts, deoxyribonucleic acid (DNA) and PCR technology have demonstrated that a nonculturable portion of the microbial community in pharmaceutical environments is viable and undetectable by compendial methods. Therefore, these new technologies provide a higher resolution and discrimination between microbial species. The new molecular methods are available that can rapidly detect microorganisms in contaminated samples. ATP bioluminescence and PCR based assays have been developed and validated for rapid microbiological screening of clinical and food samples (Hill, 1996; Ieven and Goosens, 1997). Hugo (1998) reported that ATP is the most important high-energy phosphate compound present in a microbial cell. ATP carries an important function in the microbial cell by providing the energy source to drive microbial viability and growth. ATP bioluminescence technology is based upon the

reaction of the enzyme complex luciferase–luciferin, in the presence of oxygen and magnesium, with ATP released from microbial cells resulting in the production of light. Several studies have demonstrated the applicability of ATP bioluminescence to pharmaceutical quality control and it has been previously used as an indicator of microbial viability and biomass in environmental studies (Underwood, 1998). Other studies have reported on the use of ATP bioluminescence assays for determination of the microbial content of different raw materials (De La Rosa, 1995).

Ignar *et al.* (1998) reported the detection of bacteria, yeast, and mould contamination in pharmaceutical products within 24 to 27 hours using the Celsis ATP bioluminescence system (Celsis, Inc., Evanston, IL) and specific enrichment broths. ATP bioluminescence assay provided a 24 hours count of bacteria present in water samples from a reverse osmosis/ ultrafiltration water system, hot water circulating system and cold tap water (Scalici *et al.*, 1998). ATP bioluminescence has also been used for rapid sterility testing of pharmaceutical suspensions and microbial content analysis of finished products (Ignar *et al.*, 1998). However, ATP bioluminescence provides an indication of the total microbial biomass in a product which might be including microbial species whose presence can be acceptable for non-sterile pharmaceuticals. Marino *et al.* (2000) reported that ATP bioluminescence assays have been used for rapid monitoring of quality in pharmaceutical water systems. Compared with standard methods, which require more than one growth media, the ATP bioluminescence assays were capable of detecting all microorganisms using a single growth medium in a shorter period of time. Drawback of this method is ATP extraction and detection from environmental samples is a labor-intensive and time-consuming procedure.

Several DNA molecular-based technologies are being developed primarily for the characterization of microorganisms (Olson 1998). One such system is Gene-Trak[®] (Neogen, United States), a hybridization technique used for the identification of specific organisms, even within mixed cultures, by targeting 16s or 23s rRNA. Woese (1987) identified 16s RNA as demonstrating phylogenetic relationships that could be targeted by labeled DNA probes to identify specific organisms (Stahl *et al.*, 1988). This process is facilitated by the presence of up to 10⁴ copies of ribosomal RNA within a cell, although the copy number varies depending upon the metabolic state of the organism. Gene-Trak hybridizes specific DNA probes against the rRNA of the target organism. Approximately 10⁵–10⁶ cells are required to give a positive result; thus some pre-enrichment phase is required. The protocol is generic, however, so the same methodology can be applied to a wide range of microorganisms. The method takes less than 3 hours and requires only minimal equipment. Another advantage is that considerable information is available for the nucleic acid sequences of rRNA, including those of gram-negative organisms. This means that unique DNA probes can be generated for closely related organisms such as *E. coli* and *Salmonella*.

Another technique for the isolation and detection of microorganisms from samples is the nucleic acid amplification technologies. PCR is the more familiar technique incorporating the

enzymatic amplification of target DNA sequences by using a pair of specific primers and a thermotolerant DNA polymerase. In theory PCR provides a rapid, highly sensitive (single-cell detection) and highly specific method for the isolation and enumeration of bacteria yeasts and molds. In practice, about 10^3 organisms/mL are required to ensure reliable and repeatable amplification (Fung, 1997); thus some pre-enrichment on immunomagnetic concentration may be required (Shaw *et al.*, 1998).

Because the PCR reaction is so efficient, the large amount of amplified DNA may cause cross-contamination problems that result in false positives, so good analytical technique is required. Finally, PCR is an enzymatic reaction, and it is possible that components of the sample may interfere with the amplification (Vanechoutte and van Eldere, 1997). Inhibition can be overcome by dilution or quenching of the inhibiting components (Kreader, 1996). In this following section discussed about various PCR techniques applied in pharmaceutical product analysis.

NUCLEIC ACID AMPLIFICATION TECHNIQUES (PCR)

Molecular diagnostic tests based on nucleic acid amplification technologies have become widely established in clinical microbiology laboratories in recent years, as well as in quality control (QC) laboratories for food testing and have been offered lately to the QC laboratories in the pharmaceutical sector (Denoya, 2009). Various authors performed studies on PCR based diagnosis of contaminant identification from pharmaceutical products. In this following section explained in details about various types of methods.

Uniplex PCR

Uniplex PCR used for the detection of each microorganism individually targeting the conserved region in each bacterial genome. The first PCR application to cosmetics and pharmaceutical quality control has been reported by Jimenez *et al.* (1998). They used the BAX™ system (Dupont Qualicon, Wilmington, DE), a PCR-based assay, *S. typhimurium* was detected in all 25 samples of pharmaceutical products after a 24 hours enrichment. They reported that rapid methods are faster turnover time than the standard 5 to 6 day detection time. Same author developed and validated PCR based assays to detect all other three USP bacterial indicators, *E. coli*, *P. aeruginosa*, and *S. aureus* in samples of 24 various pharmaceutical products (Jimenez *et al.*, 1999 and 2000a). They detected in artificially contaminated samples of finished products and raw materials within 27 to 30 hours.

Jimenez and Smalls (2000b) have reported the simplified method with Ready-To-Go PCR beads to detect of *B. cepacia* inartificially contaminated pharmaceutical samples. Standard methods for isolation and identification of *B. cepacia* require 5 to 6 days to be completed. However, PCR detection of samples contaminated with *B. cepacia* completed within 27 hours using the Ready-To-Go PCR beads. Apart from the bacterial contamination

in pharmaceutical products, detection of mould contamination in finished products require 6 to 8 days to be completed (Anonymous, 2007). Jimenez *et al.* (1999) developed PCR assay to detect mould contamination (*A. niger*) in pharmaceutical samples. In that study test was completed within 27 hours while the standard methods required 6–8 days. Yeast contamination, e.g., *Candida albicans*, was also detected in all the contaminated samples using a PCR-based assay (Jimenez *et al.*, 1998).

Jimenez (2000a) used a PCR-based amplification of conserved ribosomal bacterial sequences (1.5 kb); it is possible to confirm sterility. All tested samples that were positive by the PCR reaction were also positive by conventional methods. PCR-based assays reduced turn over time from 72 to 24 hours.

Simultaneous PCR detection of bacteria and mould from contaminated cosmetic/pharmaceutical samples was performed using the Stratagene Robocycler 96-Gradient. This new thermocycler allowed the simultaneous amplification of genetic sequences using DNA primers with different annealing temperatures. Low levels of microbial contamination ranging from 1 to 7 CFU were detected (Jimenez, 2001a,b). Samadi *et al.* (2007) reported PCR based detection of low levels of *S. aureus* contamination in topical lotion pharmaceutical preparations.

Recently Vijayakumar and his co-workers (2011) developed PCR to detect the low levels of *P. aeruginosa* contamination in ophthalmic viscosurgical devices by using universal and specific primers (oprL) to *P. aeruginosa* as one of the objectionable microorganisms in pharmaceutical products. Various authors reported uniplex PCR assays listed in table 1.

Multiplex PCR assays

Multiplex PCR refers to the use of PCR to amplify several different DNA sequences simultaneously (as if performing many separate PCR reactions all together in one reaction). Multiplex PCR assays have been developed and validated for environmental, food and clinical analyses (Mahony *et al.*, 1995; Brasher *et al.*, 1998). So there is need to develop a multiplex assay to amplify all relevant pharmaceutical and cosmetic microbial contaminants in one assay.

Jimenez *et al.* (2000b) developed PCR analysis for detecting low levels of bacteria and mould contamination in pharmaceutical samples using Ready-To-Go PCR. In this method samples were artificially contaminated with less than 10 CFU of *E. coli*, *S. aureus*, *P. aeruginosa* and *A. niger*. Standard methods required 6 – 8 days while PCR method detects the contaminants within 27 hours.

Farajnia *et al.* (2009) carried out a study on simultaneous detection and identification of four indicator pathogenic bacteria in a single PCR reaction in expectorant syrup. They used specific primers for four objectionable bacteria and their detection time was 27 hours. In India, a study from Hyderabad, Karanam *et al.* (2008) developed multiplex PCR for detection of indicator pathogens from various raw materials and finished products.

Table 1: Pharmaceutical products analyzed by uniplex PCR method nucleic acid amplifications by various authors (Sandle and Vijayakumar, 2014)

Test products	PCR for	Pre-enrichment step included	PCR Inhibition	Reference
Vee gum, Carboxymethylcellulose Silica, Starch, Simethicone emulsion, Antiflatulent liquid	<i>Salmonella typhimurium</i>	Yes	No	Jimenez <i>et al.</i> , (2001a)
Topical lotion	<i>S. aureus</i>	Yes	No	Samadi <i>et al.</i> , (2007)
Hydroxypropyl mehtyl cellulose and Sodium Hyaluronate	<i>P. aeruginosa</i>	No	No	Vijayakumar <i>et al.</i> , (2011)

Table 2: Pharmaceutical products analyzed by multiplex PCR by various authors (Sandle and Vijayakumar, 2014)

Test products	PCR for	Pre-enrichment step included	PCR Inhibition	Reference
Fluoride dentifrice, Medicated dentifrice, Sleeping tablet, Antiflatulent liquid, Carboxymethylcellulose, Simethicone and Lactose	<i>B. cepacia</i> <i>Bacteria and Mould</i>	Yes	No	Jimenez and Smalls (2000)
Lactose, Nicotinamide, Sodium starch glycollate, Ranitidine HCL, Mannitol, Ibuprofen suspension	<i>S. aureus</i> , <i>Salmonella</i> spp. <i>E. coli</i> <i>P. aeruginosa</i>	Yes	No	Karanam <i>et al.</i> , (2008)
Expectorant syrup	<i>S. aureus</i> , <i>Salmonella</i> spp. <i>E. coli</i> <i>P. aeruginosa</i>	Yes	No	Farajnia <i>et al.</i> , (2009)
Different pharmaceutical dosage forms and raw materials	<i>S. aureus</i> , <i>Salmonella</i> spp. <i>E. coli</i> <i>P. aeruginosa</i>	Yes	No	Regheb <i>et al.</i> , (2012)

In their study, the detection limits for artificial contaminants was 1CFU/g, whereas in the case of conventional method, the detection limit was > 2 CFU/g. Similarly, when tested with possibly contaminated samples, 35% was detected for *E.coli*, *Salmonella* spp., *S. aureus* and *P. aeruginosa* with multiplex PCR, while only 21% was detected with standard conventional microbial methods. Recently Regheb and his associates (2012) reported uniplex, duplex and multiplex PCR study for detection of the four main specified microorganisms according to the pharmacopeial recommendations, *E. coli*, *S. aureus*, *P. aeruginosa*, and *Salmonella* spp. was optimized in different pharmaceutical dosage forms and raw materials. Various authors' reports are listed table.2. Furthermore, the recent advances in DNA microchip technology can lead to specific assays for quality control purposes. DNA microchips have already been used for detecting multiple microbial populations in environmental and clinical samples (Guschin *et al.*, 1997). The same technology might be capable of detecting microbial contaminants in pharmaceutical samples.

ADVANTAGES AND LIMITATIONS

Rapid detection of objectionable microorganisms results in faster implementation of corrective actions. Detection times using PCR range from 24 to 27 hours. This is a significant reduction when compared to the standard 5 to 7 days detection time. However, drawbacks of these methods are validation and implementation of these new methods are not widely adopted by industry (Jimenez, 2004 and 2007). Some of the major concerns for implementation of PCR-based assays as routine quality control analysis in pharmaceutical laboratories are listed below.

- Absence of validation guidelines
- Uncertain regulatory status

- Lack of validation and technical support
- Lack of understanding of the technologies
- Lack of published scientific studies
- Hesitation from managers and companies to try new methods
- Lack of resources for technology evaluation and implementation
- Systems are expensive
- Underestimation by vendors of regulatory requirements

CONCLUSION

The goal of this review is to describe the different types of nucleic acid amplification techniques and methods available to pharmaceutical microbiologists working on quality control of pharmaceutical products and develop some awareness among other pharmaceutical scientists. The present review is limited to the validation work published in the peer review scientific literatures. Overall, this article has concluded that rapid methods provide reliable and cost-effective analysis for the microbiological evaluation of pharmaceutical products.

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How to cite this article:

Mohammad Saleh Al-Aboody. Review on Application of Nucleic Acid Amplification Techniques in Pharmaceutical Products Analysis. *J App Pharm Sci*, 2015; 5 (12): 154-158.