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Research Article

Molecular diagnosis of *Pseudomonas aeruginosa* contamination in ophthalmic viscosurgical devices

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ABSTRACT

Polymerase chain reaction (PCR) was performed to verify the utility of this technique in detecting low levels of microbial contamination in quality control of ophthalmic viscosurgical devices (OVDs). Universal and specific primers (oprL) were applied to identify *Pseudomonas aeruginosa* as one of the objectionable microorganisms in pharmaceutical products. Samples of hydoxypropylmethylcellulose, sodium hyaluronate inoculated with defined number of *Pseudomonas aeruginosa* cells and subsequently exposed to boiling for 15 minutes for releasing DNA materials from the contaminants. The treated samples were subjected to PCR amplification. Agarose gel electrophoresis revealed amplified fragments as predicted, with no interference from the products and other environmental bacterial and fungal strains included in the study. The minimum detection limit of *Pseudomonas aeruginosa* DNA was 2.1 ng. In contrast to conventional culturing methods that require mean of 5 – 6 days for identification of *Pseudomonas aeruginosa*, the entire mentioned PCR assay lasted about 4 – 5 hours.

Keywords: Polymerase chain reaction; *Pseudomonas aeruginosa*; contamination; OVDs.

INTRODUCTION

At the beginning of the 21st century, microbial contamination of non-sterile products is one of the major reasons for product recalls, production shutdowns, and losses in labor and manufacturing. Huge economic lost is due to the lack of quality control, process control, and proper testing. This could result in a reduced shelf life by compromising product integrity or present potential health hazard to the consumers. (Jimenz, 2004, Mestrandrea, 1997). When a contaminant is found in a production batch, an investigation is rapidly started to determine the contamination source, the numbers, and the types of microorganisms. For this purpose, United States Pharmacopeia's microbial limits tests require should absence of 4 different bacterial indicators like *Salmonella* sp. *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli* from the pharmaceutical products (USP, 2006). In spite of regulatory guidelines and recommended methods, microbial contamination is still one of the major causes for products recalls worldwide. Of four USP and EP (European Pharmacopoeia) bacterial indicators, *S. aureus, P. aeruginosa*, and *E. coli* were found in samples of toothpastes, topical products, shampoos, oral solutions, drugs, and disinfectants (Berkelman et al, 1984, Kallings et al, 1966, Mitchell & Hayward, 1966, Robinson, 1971). Among these, *Pseudomonas aeruginosa* is the most frequently found in pharmaceutical products from all over the world (Jimenz, 2007).

Pseudomonas aeruginosa is a gram-negative, rod-shaped obligate aerobic bacterium with minimal nutritional requirements. It is often found in moist environment and can cause infections in immunocompromised or otherwise susceptible hosts. Numerous outbreaks have been associated with faulty or unclean medical equipments or products, contamination from personnel or environmental reservoirs (Cobben et al, 1996, Schelenz & French, 2000, Iversen et al, 2007).

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 pharmacopeias but all are traditional methods of identification based microbiological culture methods like morphological and biochemical characterization of microorganisms. This conventional methods are very laborious and time consuming and not always specific enough to discriminate among species and strains (Denyer et al, 2004). 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.

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The application of rapid methods like polymerase chain reaction (PCR) assays to food testing and clinical analysis has resulted in faster quality evaluation of foods and rapid diagnosis of pathogenic conditions (Hill, 1996, Crothchfel et al, 1997). However, PCR applications for cosmetic and pharmaceutical quality control have been rather slow. While few studies have been devoted to application of rapid technologies in elucidating the presence of microbes in pharmaceutical preparations (Jimenez et al, 2000, Jimenez et al, 2001, Karanam et al, 2008). The polymerase chain reaction is a technique that amplifies specific segments of DNA of contaminants. In our literature search there were no reports available about application of rapid method to detect contaminants from ophthalmic viscosurgical devices (OVDs). The OVDs have significantly helped advance ophthalmic surgical techniques and improve patient outcome. Hydroxypropyl methylcellulose (HPMC) and sodium hyaluronate are the example of OVDs.

Hence, the aim of the present study is to develop a PCR assay to detect culprit *Pseudomonas aeruginosa* in hydroxypropyl methylcellulose and sodium hyaluronate.

MATERIALS AND METHODS

Microorganisms

Pseudomonas aeruginosa ATCC 27853 was used in this study. The specificity of the DNA based test for identification of *P. aeruginosa* was verified with other reference strains include *Staphylococcus aureus ATCC 25923, E. coli ATCC 25922* and *Salmonella abony NCTC 6017.* The specificity was also checked with pharmaceutical environmental bacterial isolates like *Bacillus sp., Staphylococcus sp.,* and *Micrococcus sp*., and fungal isolates like *Aspergillus niger, Penicillium sp.,* and *Cladosporium sp*.,

Sample Preparation

Ophthalmic viscosurgical devices (2 % Hydroxypropyl methylcellulose and 1.4% sodium hyaluronate, Aurolab, India) were selected as the test pharmaceutical product. The bacterial inoculum was prepared by suspending overnight colonies from Muller-Hinton Agar medium in 0.9% saline. The inoculum was adjusted photometrically at 600 nm to a cell density equivalent to approximately 0.5 McFarland standard (1.5 x 10^8 cfu/ml). Equal volume of test sample was inoculated with bacterial suspension to a final concentration about 0.75 x 10^8 cfu/ml. This bacterial concentration was verified by serial dilution and plate count methods.

DNA Extraction

From artificially contaminated samples, 1 ml aliquot was added into sterile eppondorf tube and placed in water bath at 95^0 C for 15 mins. This was considered as positive product control. Un-inoculated viscosurgical devices were placed in same condition and considered as negative product control. DNA was extracted by following kit extraction method (Qiagen, Germany) from overnight grown *P. aeruginosa* culture. This DNA sample was considered as positive control. The purity of the DNA was checked by 0.8% agarose gel electrophoresis and the quantity of the extracted DNA was determined by nanophotometer (Implen, Germany)

PCR amplification

P. aeruginosa specific primers synthesized (Forward 5'-CGGGCGTGCTGATGCTCGTAT-3', Reverse 5'-GCGCGAG GAACGTCAGGACAC-3') and were obtained from sigma, Bangalore, India. The primers are highly conserved region of outer cell membrane lipoprotein L gene (oprL) of *P. aeruginosa*, they can amplify a 709 bp product.

The reaction mixture (50 µl) consisted of 5 µM each forward and reverse primers, 200 µM deoxynucleoside triphosphate, 10 µl of the lysate as DNA template, 1.0 U of Taq DNA polymerase (Genei, Bangalore) and the manufacturer supplied 10X PCR buffer (10mM tris/HCl, 50 mM KCl, 1.5 mM $MgCl_2$). Negative control reactions containing distilled water instead of DNA templates were also included throughout the PCR assay to avoid false positive results.

PCR was performed in the thermocycler (Eppendorf master cycler proS, Germany) with initial denaturation at 95^0C for 5 minutes, followed by 40 cycles at 95, 55 and 72^0C for 30 seconds, 30 seconds and 1 minute respectively, and a final extension at 72^0C for 7 minutes.

Determination of sensitivity and specificity

For the purpose of estimating minimum detection limit of *P. aeruginosa* DNA, pure DNA sample (140 ng/µl) was diluted in sterile distilled water to prepare series of two fold decreasing DNA concentrations of *P. aeruginosa* upto 0.5 ng/µl. Minimum DNA, 3 ng/µl was spiked with samples of hyrodoxypropylmethyl cellulose and sodium hyaluronate and performed PCR. The PCR primers of *P. aeruginosa* were also examined for their specificity against reference stains and pharmaceutical environmental contaminants.

Agarose gel electrophoresis

Agarose gel electrophoresis was carried out at 150 V for 30 min using 1.5% agarose gel (Himedia) containing 0.5 µg/mL of ethidium bromide. After gel electrophoresis, photographs were taken using a UV gel documentation System (Wilber Lourmat, France). 100 bp DNA ladder (Genei, Bangalore) was used for determining the size of the amplified products. A positive result (*P. aeruginosa* specific band) was indicated by a fluorescent band at the 709 bp level.

RESULTS

Uniplex PCR was employed to detect *P. aeruginosa* from artificially contaminated ophthalmic viscosurgical devices. As shown in figure 1 the expected 709 bp DNA

fragment was successfully amplified with oprL primers for *P. aeruginosa*. Direct boiling for 15 minutes of product with artificially contaminated *P. aeruginosa* yielded 38 ng/µl which was equivalent to the cell density 0.75 x 10^6 CFU. The sensitivity of the assay was determined to be about 140 – 0.5 ng of *P. aeruginosa* DNA and detected upto 2.1 ng (Figure .2)

In figure 3 PCR products a single band in the expected size (709 bp) was observed without any non specific PCR band against common bacterial and fungal contaminant DNA of pharmaceutical isolates. These results were indicated 100% specific to *P. aeruginosa* detection.

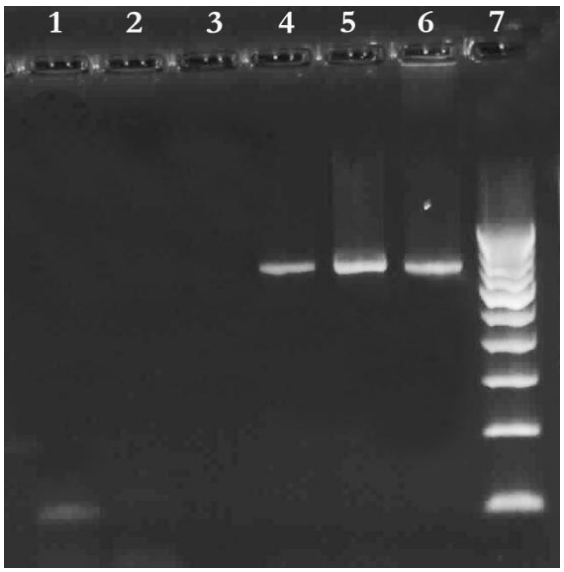

Figure 1: PCR detection of P. aeruginosa contamination using different spiked samples

Lane 1 – Negative control; Lane 2 – Hydroxyprophyl methyl cellulose (un-inoculated); Lane 3 – Sodium Hyaluronate (un-inoculated); Lane 4 – *P. aeruginosa* spiked with HPMC (709 bp); Lane 5 – *P. aeruginosa* spiked with sodium Hyaluronate (709 bp); Lane 6 – Positive control 709 bp product (*P. aeruginosa)*; Lane 7 – 100 bp DNA ladder

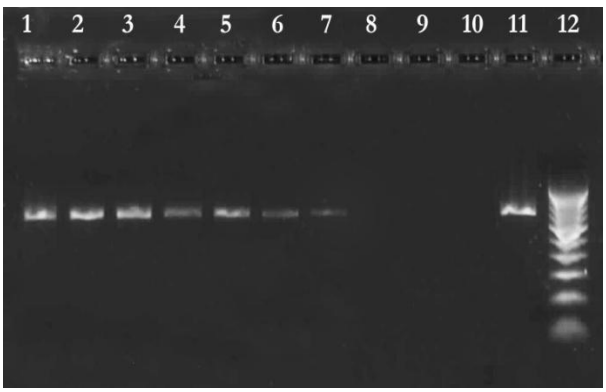

Figure 2: Minimum detection level of P. aeruginosa DNA quantity by PCR

Lane 1 – 7; 140,70,35,17.5,8.75,4.32,2.1 ng; Lane 8 & 9: 1 and 0.5 ng; Lane 10: Negative control; Lane 11: Positive control 709 bp product (*P. aeruginosa)*; Lane 12 – 100 bp DNA ladder.

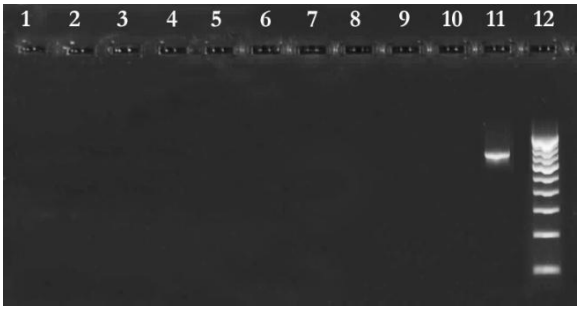

Figure 3: Specificity of PCR against reference strains and environmental contaminants

Lane – 1 to 3: Reference strains of *Staphylococcus aureus, E. coli, Salmonella abony,* Lane 4 – 6: Environmental isolates of *Micrococcus* sp., *Bacillus* sp., *Staphylococcus* sp., Lane 7 – 9: Environmental isolates of *Aspergillus niger, Penicillium sp., Cladosporium* sp., Lane 10 – Negative control; Lane 11 – Positive control 709 bp product (*P. aeruginosa),* Lane 12 – 100 bp DNA ladder

DISCUSSION

P. aeruginosa is a Gram-negative microorganism, inherently resistant to common antibiotics and it is able to grow in some eye drops (especially quaternary ammonium compounds), saline, and other aqueous solutions (Zhou et al, 2007). The presence of Gram negative bacteria might indicate lack of process control in water systems and raw materials. The standard methods are performed to quantify, detect, and identify the numbers and types of microorganisms present in a given pharmaceutical batch. These methods are based upon the enrichment, incubation, and isolation of microorganisms from pharmaceutical samples. As per pharmacopeial guidelines, the selective medium for *P. aeruginosa* is cetrimide agar or *Pseudomonas* agar. Because of the long incubation times, continuous manipulation, and time-consuming procedures, results are normally obtained within 5 – 7 days. It has been recently reported that standard methods underestimate the microbial communities present in pharmaceutical environments (Kawai et al, 2002, Nagarkar et al, 2001, Kawai et al, 1999, Venkateswaran et al, 2003). To manage with required microbial limit tests, there is always a need for a rapid, sensitive and efficient method to take rapid corrective actions to prevent further contamination of production samples, huge financial losses, and release of contaminated product that can cause disease to consumers.

In our study, primers of outer membrane lipoprotein gene (oprL) were used to detect *P. aeruginosa* contamination in pharmaceutical samples. The polymerase chain reaction conditions specific for *P. aeruginosa* detection were employed in the samples of hydroxypropyl methylcellulose and sodium hyaluronate. These ophthalmic viscosurgical devices are essential tools for

Table 1: Pharmaceutical products analyzed by PCR methods by various authors

Test products	PCR for	Pre-enrichment step included	PCR Inhibition	Reference
Fluoride dentifrice Medicated dentifrice Sleeping tablet Antiflatulent liquid Carboxymethylcellulose (CMC) Simethicone Lactose	*Burkholderia cepacia*	Yes	No	Luis Jimenez and Stacey smalls, 2000
- do -	*Bacteria & Mold*	Yes	No	Jimenez L et.al., 2000
Vee gum, Carboxymethylcellulose (CMC) Silica, Starch, Simethicone emulsion, Antiflatulent liquid	*Salmonella typhimurium*	Yes	No	Jimenez L et.al., 2001
Topical lotion	*Staphylococcus aureus*	Yes	No	Samadi N et.al., 2007
Lactose, Nicotinamide, Sodium starch glycollate, Ranitidine HCL, Mannitol, Ibuprofen suspension	*S. aureus,* *Salmonella,* *Escherichia coli* *P. aeruginosa*	Yes	No	Karanam V. R., et.al., 2008
Expectorant syrup	*S. aureus,* *Salmonella* *E. coli* *P. aeruginosa*	Yes	No	S. Farajnia et. al., 2009
Hydroxypropyl mehtyl cellulose Sodium Hyaluronate	*P. aeruginosa*	No	No	Present study

the anterior segment surgeon and routinely used for intraocular surgery. Rapid detection and identification of bacteria, yeast, and mold using PCR technology have been reported in the food industry and clinical laboratories (Ieven, 1997). PCR analysis can provide rapid quality evaluation of foods and pathogen detection leading to faster corrective actions. Application of PCR to cosmetic and pharmaceutical quality control has been reported in various samples for the detection of bacteria and mold (Hill, 1996, Jimenez et al, 2000, Jimenez et al, 2001).

Sample preparation prior to PCR analysis can be the most limiting factor during development and optimization of a given PCR assay (Jimenez et al, 1999). To overcome PCR inhibition problems and to increase the sensitivity of the assay, pre enrichment methods were used by various authors (Jimenez et al, 1999 & 2001, Samadi et al, 2007, Karanam et al, 2008, Farajnia et al, 2009,). After the enrichment step, sufficient bacteria were grown and allowed the pathogens to be detected by PCR when the original sample had < 1 CFU/mL. As per their studies, the samples were spiked with microbial levels of < 10 and < 100 cfu/g or mL of microorganisms. After 24 hours enrichment, the assay's sensitivity ranged from 10^4 to 10^5 cells/ mL (Jimenez et al, 2001). None of the assays showed false-positive results. No inhibitory effects were reported by any of the studies, which indicate that the sample dilution during the pre-enrichment step might help to overcome the effect of inhibitory substances (Table.1).

The present study is directed towards the evaluation of a simple lysis protocol coupled with speedy PCR system for detection of low levels of *P. aeruginosa* in hydroxypropyl methylcellulose and sodium hyaluronate. The pre enrichment step was not included in the study and simple DNA extraction method was applied to artificially contaminated viscosurgical devices, which were boiled at 95^0 C for 15 minutes to release the DNA materials. The small volume of treated product (10 µl) taken for PCR reaction had no inhibitor effects and all the positive cultures showed 709 bp product. There was no lysis buffer with proteinase K used in this study for DNA extraction. This evident that DNA extraction from the ophthalmic pharmaceutical samples requires less stringent conditions and lack of inhibitory substances such as those found in food and clinical samples. However, the sample pre enrichment is the most important step before DNA extraction to increase sensitivity of <10 CFU/gm or mL of sample. In the PCR assay of the present experiment, the distinct 709 bp fragment formed for all artificially contaminated products with *P. aeruginosa*.

Even though OVDs are available in sterile form, it should be pure, atoxic and endotoxin free. Endotoxins are lipopolysaccarides released from the outer cell wall after Gram negative bacterial cell lysis. If *Pseudomonas*

contamination present in the raw material or get contaminated in the production process while OVDs manufacturing will cause the elevated level of endotoxin in the product after sterilization process. A rapid method of PCR assay could be an effective analysis in-process samples while preparing the viscosurgical devices instead of 7 days conventional culture based microbial limit tests in QC program. Total time consumed for present test method is 4 – 5 hours while excluding a pre enrichment step, it is contrary to other available reports, because they included the preenrichment step and time required for PCR based detection reported as 34 – 30 hours (Jimenez et al, 2001, Karanam et al, 2008).

This is the first study report showing OVDs are not having PCR inhibitor property and quits the DNA extraction by pre enrichment step. However, pre-enrichment will help to increase the sensitivity and avoids the inhibition of PCR conditions test but could take additional time. The similar type of PCR based study has been conducted by Samadi et al, (2007) to detect *Staphylococcus aureus* contamination in topical lotions, but they followed enrichment step.

In this study the sensitivity of the minimum detection limit of DNA quantity of *P. aeruginosa* is 2.1 ng. This is comparable with other available reports (Jimenez et al, 2000) the sensitivity of the detection limit by utilizing minimum quantity of DNA 1.56 ng. The specificity of the primers used in the *P. aeruginosa* identification revealed the absence of non-specific amplification with other environmental bacterial contaminants like species of *Staphylococcus, Micrococcus, Bacillus* and fungal contaminants like species of *Aspergillus, Penicillium* and *Cladosporium*. This showed that the primers used in this study are highly specific to detect *P. aeruginosa* from the samples. The similar primers were already used in multiplex PCR by Karnam et al., and their specificity results are comparable with our results (Jimenez et al, 2000).

CONCLUSION

Based on our findings, it is inferred that the PCR assay provides a sensitive and highly specific method for *P. aeruginosa* detection at low levels (upto 2.1 ng of DNA quantity) in the ophthalmic viscosurgical devices like hydroxypropyl methylcellulose and sodium hyaluronate. The OVD's did not possess the inhibitory property in PCR. This method helps for the optimization of product manufacturing, and quality control of products in a timely manner (within 6 hours) when compared to conventional culture methods. And also, this PCR method can be further adapted to other cosmetics and pharmaceutical products for rapid identification of dangerous contaminant *P. aeruginosa*.

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