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MOLECULAR VERSUS CONVENTIONAL TECHNIQUES FOR THE DETECTION OF *STAPHYLOCOCCUS AUREUS, PSEUDOMONAS AERUGINOSA* AND *CANDIDA ALBICANS* IN NON-STERILE PHARMACEUTICAL PREPARATIONS

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Keywords:

Non-sterile pharmaceutical preparations, Topical indicator pathogens, Conventional detection, Monoplex and Multiplex PCR

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ABSTRACT: Notable progress has been made in methods that encourage the use of PCR as a rapid and accurate tool in quality evaluation of pharmaceuticals. In this study, monoplex and multiplex PCR based assays were developed and compared with standard conventional methods for rapid detection of three specified topical indicator pathogens, *Pseudomonas aeruginosa*, *Staphylococcus aureus,* and *Candida albicans,* in nonsterile pharmaceutical preparations. The detection limit of monoplex PCR assays for the microbial targets was achieved at 100 fg purified DNA and 10 CFU/ml for *Pseudomonas aeruginosa,* and 1fg purified DNA and 10 CFU/ml for each of *Staphylococcus aureus* and *Candida albicans.* No change in the detection limit for CFU/ml of the three tested indicator pathogens was obtained upon using mPCR assays. The results of applying both conventional and PCR detection methods for different cream and lotion preparations revealed a 100% correlation between both methods. The PCR based detection method can be completed in 8 h versus 5-6 days in case of conventional methods, but the former can't differentiate between viable and dead cells. PCR assays can be used efficiently and in a cost-effective manner to exclude the contamination of pharmaceutical products by the indicator pathogens. Even though, in case of contamination by non-viable indicator organisms, PCR technique can still be used after partial incubation of cultivated test sample. Thus, PCR assays provide specific, reliable results that can be incorporated in the quality evaluation of pharmaceuticals and will impact positively in terms of cost and time.

INTRODUCTION: The presence of microbial contaminants was not only found to cause physicochemical changes that led to the spoilage of numerous products [1-3] but were also proved to be a potential health hazard to the consumer [4-6] as documented by a large number of incidents of drug-related infections [1, 7, 9].

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Nonsterile pharmaceutical products are susceptible to microbial contamination during both their manufacture and use [10,] and this has compelled official compendia to develop limits and tests for different aspects of microbial contamination and preservative efficacy [11]. Topical preparations, although not required by most Pharmacopoeia to be sterile, are, none the less, required to pass microbial limit tests and tests for the absence of specified indicator micro-organisms, *Pseudomonas aeruginosa, Staphylococcus aureus* and *Candida albicans* which can be regarded a hazard to consumers and indicative of contamination [12].

The detection of microbial contaminants in pharmaceutical preparations has been generally conducted using standard cultivation-based methods as depicted in many studies [13-17]. The conventional detection methods, despite their advantages and widespread use, yet are somewhat labour intensive, time consuming, not always specific and discriminate poorly among species and strains [18]. Moreover, they offer poor detection of slow growing or viable but non-culturable (VBNC) microorganisms [19, 20]. Nevertheless, pharmaceutical companies have relied on these methods for quality evaluation of raw materials and finished products. However, continuous improvement of current pharmaceutical products requires a faster methodology for quality control purposes that will be fast, sensitive, accurate, and cost effective [18].

Molecular biology-based microbial detection systems have been making notable progress in the clinical and food microbiology sectors and are now also being used in pharmaceutical applications [21]. There is a growing trend that more and more pharmaceutical companies are implementing genetic techniques, such as polymerase chain reaction (PCR), for microbial identification and detection applications due to their greater accuracy, ease of use, speed to result, low cost as compared with classical methods [22, 23]. These methods also offer a suitable approach to the detection of slow-growers and/or viable non-culturable (VNC) microorganisms [24, 25]. Further advancements in PCR methods is the multiplex PCR, which involves simultaneous amplification of more than one target gene in a single reaction.

Several studies on using PCR for the microbiological quality control of pharmaceutical products have been published to date. A commercial system, the BAXTM, has been validated for detecting Salmonella typhimurium in pharmaceutical environments [26]. PCR assays have also been developed for detecting *S. aureus, P. aeruginosa, E. coli, Aspergillus niger* and *Clostridium perfringens* [18, 27-29]. MPCR assays have been developed for the detection of four indicator pathogens [30, 31]. However, no PCR assay has been reported for the simultaneous detection of topical indicator pathogens in pharmaceutical environments. The objective of the present study was to develop monoplex and multiplex PCR assays for the detection of specified indicator pathogens in nonsterile pharmaceutical products. The study also involved testing low microbial levels at which these assays will be valid as well as comparing such assays with standard conventional methods.

MATERIALS AND METHODS:

Microorganisms and Culture Media:

Contaminants from Pharmaceutical Preparations: In a previous study, 60 bacterial and 31 fungal isolates were recovered from pharmaceutical preparations [32]. Of these, 6 bacterial isolates and one fungal isolate were selected for completing the present study.

Reference Strains and Clinical Isolates: Reference strains and clinical isolates used in this study included; *Staphylococcus aureus* ATCC 433001, *Escherichia coli* ATCC 25922 and one isolate each of *Salmonella enterica*, *Pseudomonas aeruginosa, Candida albicans, Heamophilus influenzae, Enterococcus* spp., *Proteus* spp., *Streptococcus pyogenes, Streptococcus pneumoniae, Staphylococcus epidermidis, Streptococcus viridians, Bacillus subtilis*. The strains were obtained from German University in Cairo, Alfa Laboratories in Egypt, and the National Research Center in Egypt (personal communication). The clinical and reference strains were used to establish conditions and validation of monoplex and multiplex PCR assays. All of the bacterial strains, except *Streptococcus* spp. and Heamophilus influenzae were cultured on nutrient agar slants at 37 °C for 24 h. *Streptococcus* spp. were grown on blood agar plates whilst Haemophilus influenzae was grown on chocolate agar plates. Fungal isolates were cultured on Sabouraud Dextrose Agar slants (SDA) at 30-35 °C for 24-48 h.

Artificially and Possibly Contaminated Pharmaceutical Samples: For monitoring the safety of topical non-sterile pharmaceutical preparations, the USP microbial limit tests require the absence of three specified microbial indicators; *Staphylococcus aureus, Pseudomonas aeruginosa* and *Candida albicans*. Testing for these specified microbial contaminants using conventional techniques was conducted in an earlier study [32], where a total of 280 possibly contaminated non-sterile pharmaceutical samples were tested.

The results of such were further confirmed in the current study using molecular techniques. Moreover, in this study, representative non-sterile pharmaceutical preparations indicated for topical application; a cream and a lotion formula, were artificially inoculated with different bioburdens of *Pseudomonas aeruginosa, Staphylococcus aureus* and *Candida albicans* to assess the influence of an active substance and other constituents on the isolation of microbial DNA, amplification and minimum detection limit of PCR. For PCR, not less than 1 gm or 1 ml of the tested pharmaceutical product was used. Sample preparation was conducted according to the United States Pharmacopoeia [11] as previously described [32].

Test for Specified Microbial Contaminants using Conventional Techniques: Testing conducted in an earlier study [32], revealed the recovery of the three bacterial indicators from seven products (five *S. aureus* isolates coded 1C, 2G, 3N, 9T and 17S were isolated from a cream, a gel, an ointment, a tablet, and a syrup, respectively; one *Pseudomonas aeruginosa* isolate coded 10C, was recovered from a capsule and one *Candida albicans* isolate, coded 9S, was recovered from a syrup).

Identification of the Specified Microbial Contaminants using Molecular Techniques: The seven isolates (6 bacterial and one fungal isolate) suspected to be of the topical USP indicator pathogens were identified by the application of polymerase chain reaction using specific primers for each suspected pathogen.

Isolation of Genomic DNA:

Preparation of Purified DNA: Genomic DNA of each suspected isolate was extracted from overnight cultures of the tested bacterial isolate grown in TSB using the Gene-jet purification kit (fermentas, Lithuania) according to the manufacturer's instructions. The DNA was eluted from the column with two 100 µl aliquots of the elution buffer, supplied with the kit, and the eluates were then combined. Eluted DNA was stored at −20 °C. Aliquots (10 µl) of the eluted DNA were used in 25 µl PCR mixture. The purity of the DNA was checked by agarose gel electrophoresis and the quantity of extracted DNA was detected spectrophotometrically (UV-VIS Spectrophotometer 6800 Jenway) at wave-length 260 nm according to Sambrook and Russell [33].

Rapid Preparation of DNA Extracts: Genomic DNA was also extracted from the microbial cultures by the boiling-centrifugation method with some modifications, as described previously [34, 35]. Aliquots of overnight cultures were centrifuged at 1400 rpm for 10 min. The supernatant was discarded, and the pellets were suspended in 300 µl of H_2O by vortexing. After centrifugation at 1400 rpm for 5 min, the supernatant was again discarded, and the pellets were suspended in 100 µl of molecular grade water by vortexing and heated at 100 °C in a water bath for 15 min. The lysate was immediately chilled on ice for 5 min, centrifuged at 1400 rpm for 10 min and the supernatant was used as a template for PCR detection.

PCR Primers: Primers were selected on the basis of published nucleotide sequences. The nucleotide sequences of all PCR primers used in this study, their target genes, and the sizes of the respective amplicons produced are listed in **Table 1**. Specific primers for amplification of certain bacterial genes as well as ribosomal 16S rRNA primers (a universal primer for detection for any bacteria) were applied. Detection of *Pseudomonas aeruginosa* was based on the amplification of the 504 bp segment of the outer membrane lipoprotein (oprl) gene, specifically for *Pseudomonas aeruginosa* [36].

TABLE 1: PCR PRIMERS USED IN THE STUDY

Indicator pathogen	Target gene	Primer	Primer sequences (5' to 3')	Annealing Temp. (Ta)	Amplicon Size (bp)
Staphylococcus aureus	*Nuc*	nuc-F	GCGATTGATGGTGATACGGTT	55°C	270
		nuc-R	AGCCAAGCCTTGACGAACTAAAGC		
Pseudomonas aeruginosa	*OprL*	PAL-F	ATGGAAATGCTGAAATTCGGC	57°C	504
		PAL-R	CTTCTTCAGCTCGACGCGACG		
Candida albicans	25S rRNA	CAL5	TGTTGCTCTCTCGGGGGCGGCCG	58°C	175
		NL4CAL	AAGATCATTATGCCAACATCCTAGGTA		
Universal primer	16S rRNA	DG74	AGGAGGTGATCCAACCGCA	55°C	375
		RW01	AACTGGAGGAAGGTGGGGAT		

Detection of *Staphylococcus aureus* was based on the amplification of 270 bp segment of the thermostable nuclease gene, previously shown to be specific for *Staphylococcus aureus* [37, 35]. Detection of *Candida albicans* was based on the amplification of 175 bp fragment of 25S rRNA gene of *Candida albicans* [38]. The pairs of the universal primer, derived from highly conserved regions of the bacterial 16S rRNA gene, were used as a positive control since they can amplify a 375 bp product from any bacterial species [39]. All oligonucleotide primers used in this study were synthesized by Fermentas, Lithuania.

Reaction mixture and working PCR protocol for monoplex PCR: PCR amplification was carried out in a reaction volume of 25 µl. DNA was amplified according to reaction conditions published for each primer pairs [36-39] with slight modifications. PCR mixtures contained 1.5 U Taq DNA polymerase (Thermo Scientific Maxima Hot start Taq DNA polymerase 5 U/µL, Fermentas), 1X Hot Start PCR buffer (200mM Tris-HCl, 200 mM KCl, 5mM $(NH_4)_2SO_4$), 200 µM of each dNTP (Fermentas), 0.6 µM of each primer pair, 1.5 mM $MgCl_2$ (2.5mM $MgCl_2$ for Candida albicans) and 10 µl of extracted DNA. PCR was conducted in a Perkin Elmer 480 Cetus DNA thermal cycler (USA) with initial denaturation at 95°C for 4 min followed by 30 cycles of amplification consisting of denaturation at 95°C for 30 seconds, annealing at specified temperature **Table 1** for 30 seconds and extension at 72°C for 30 seconds with a final extension step at 72°C for 10 min. Positive control consisted of DNA isolated from the corresponding reference microbial strain grown in TSB (bacteria) or SDB (Candida albicans) while negative controls consisted of PCR mixtures with primers, but without DNA template.

Agarose Gel Electrophoresis: Aliquots (5 µl of genomic DNA or 10 µl of PCR products) were mixed with 6X DNA loading dye (Fermentas, Lithuania) and electrophoresed using horizontal 1.5 % w/v (for PCR products) or 0.8 % w/v (for genomic DNA) agarose gel, containing 0.5 mg/ml ethidium bromide. Electrophoresis was carried out in 1x TAE buffer at 5 v/cm, and gels were visualized and photographed on a transilluminator (Fotodyne FOTO/prepI UV transilluminator, Fisher Scientific). Standard DNA molecular weight marker, 100 bp Plus DNA ladder (Fermentas, Lithuania) was included in each run.

Testing of Interferences in PCR Assays by Other Organisms: Studies were performed to validate the specificity of the primer pairs and the reaction conditions of PCR. Each primer pair was tested by PCR on separate DNA templates prepared from a panel of 13 different representative isolates previously mentioned. Genomic DNA of the different tested organisms was extracted from their corresponding cultures by boiling centrifugation method, and 10 µl aliquots of the produced supernatants were used as templates in PCR using the same reaction conditions described earlier. In the case of bacterial strains, a universal primer targeting eubacterial 16S rRNA was included in each reaction serving as an internal control. PCR mixtures (25 µl) contained 2 U Hot start DNA Taq polymerase, 1 X hot start PCR buffer, 200 µM of each dNTP, 0.6 µM of each primer pair, and 2.5 mM $MgCl_2$.

Detection Limit of PCR Assay for the Purified Genomic DNA of the Specified Tested Organisms: The detection limit of PCR was evaluated by testing different dilutions of purified genomic DNA of reference strains of the indicator pathogens. Purified genomic DNA was extracted using the Gene-jet extraction kit, and the concentration was determined spectrophotometrically (UV-VIS Spectrophotometer 6800 Jenway). DNA extracts were serially diluted 10 folds in sterile distilled water to give the concentration range of 100 ng/µl to 1 fg/µl and 1 µl aliquots of each dilution were used in PCR assays using the same reaction conditions described earlier. PCR products were subjected to agarose gel electrophoresis, and the minimum concentration of genomic DNA showing a positive signal was recorded.

Detection Limit of PCR Assay using Whole-Cell Lysates of the Specified Tested Organisms: The detection limit of PCR in terms of the minimum number of microbial cells that could be detected was also evaluated. Overnight cultures of reference strains of the indicator pathogens, adjusted spectrophotometrically at 640 nm to a final optical density corresponding to 10^8 CFU/ml were serially diluted ten-folds with sterile saline to microbial counts varying from 10^4-10^0 CFU/ml.

The microbial cell count was verified by the plate count technique. Aliquots (1 ml) of each dilution was subjected to DNA extraction by the boiling centrifugation technique described earlier with minor modification where the cell pellet product after centrifugation was suspended in 30 µl of nuclease-free water prior to boiling. An aliquot (10 µl) from DNA extract of each dilution was subjected to DNA amplification using the same reaction conditions described previously. PCR products were electrophoresed, and the minimum CFU that produced a detectable PCR band was determined.

Detection Limit of PCR Assays using Artificially Inoculated Pharmaceutical Preparations with the Specified Tested Organisms: The detection limit of PCR was examined for pharmaceutical preparations separately inoculated with different dilutions of indicator pathogens. Samples (1 g each) of each representative product were tested by preparing 1:10 sample dilutions of the product in TSB. Replicates of a cream formula were inoculated with *Pseudomonas aeruginosa* and *Candida albicans* separately, while a lotion formula was inoculated with *S. aureus* to achieve final counts of 10^4 - 10^0 CFU/ml. Aliquots (1 ml) of each dilution of the inoculated preparations were used for enumeration by plate count technique.

In parallel, equal volumes were subjected to genomic DNA isolation by the two techniques, but using reduced volumes (30 µl) for elution. The DNA extracts (10 µl each) were used as the templates for PCR amplification using the conditions described previously, and PCR products were electrophoresed using 1.5% w/v agarose gel. The detectable PCR bands corresponding to the different tested dilutions were determined.

Detection Limit of PCR Assays using Artificially Inoculated Pharmaceutical Preparations with Specified Tested Organisms Together with Other Co-existing Ones: This assay was conducted to ensure the specificity and sensitivity of the applied method in detecting low counts of indicator pathogens in pharmaceutical preparations in the presence of other pharmaceutical contaminants. Replicates of cream and a lotion formula were separately inoculated with three microbial mixtures consisting of *S. aureus* ATCC 43001 or *Pseudomonas aeruginosa* or *Candida albicans*, each mixed with the three identified bacterial contaminants to achieve final counts of $10\text{-}10^0$ CFU of each organism per ml. Genomic DNA was isolated using the Gene-jet purification kit and purified DNA extracts (10 µl each) were used as templates for PCR using conditions described previously. The PCR products were electrophoresed using 1.5% w/v agarose gel and the detectable PCR bands corresponding to the different tested dilutions were determined.

Detection of Specific Microbial Contaminants using mPCR: The optimal conditions for each monoplex PCR assay studied before were used initially in mPCR, and according to the results obtained, the conditions were modified for optimization. This was carried out by testing variable concentrations of some PCR components (primer, $MgCl_2$, and buffer) as well as testing different reaction conditions (annealing temperatures, number of cycles, annealing and extension times). The optimized reaction mixture contained 1 X Hot start PCR buffer, 200 µM of each dNTP, 0.4 µM of nuc-F/nuc-R, 0.7 µM PAL-F/PAL-R, 0.3 µM CAL5/NL4CAL, 3.5 mM $MgCl_2$ and 2.5 U Hot Start Taq DNA polymerase.

PCR was conducted in a Perkin Elmer Cetus DNA thermal cycler with an initial denaturation at 95 °C for 4 min followed by 40 cycles of amplification consisting of denaturation at 95 °C for 30 sec, annealing at 56 °C for 1.5 min and extension at 72 °C for 1 min with a final extension step at 72 °C for 10 min.

Detection Limit of mPCR Assay using Mixed Cell Cultures of the Three Specified Tested Organisms: Overnight enumerated cultures of *Staphylococcus aureus, Pseudomonas aeruginosa,* and *Candida albicans* were mixed together at equal counts per the applied volume, and the obtained suspension was ten-fold serially diluted in sterile saline to a microbial count varying from 10^4- 10^0 CFU of each pathogen/ml. An aliquot (1ml) of each dilution was subjected to genomic DNA extraction. PCR amplification and gel electrophoresis using the conditions previously described, and the minimum concentration showing a detectable band was recorded.

Detection Limit of mPCR using Artificially Inoculated Pharmaceutical Preparations with Mixed Cultures of the Three Specified Tested Organisms: In order to verify the validity of mPCR method for detection of pathogens in pharmaceutical preparations, a lotion, and a cream formula were separately inoculated with mixed cultures of *S. aureus* ATCC 43301, *Pseudomonas aeruginosa*, and *Candida albicans* to achieve final counts of 10^4-10^0 CFU of each of the 3 indicator pathogens per ml.

An aliquot (1ml) of each dilution was subjected to genomic DNA extraction, PCR amplification, and gel electrophoresis using the conditions previously described. The minimum concentration showing a detectable band was recorded.

RESULTS:

Detection of *S. aureus*, *Pseudomonas aeruginosa*, and *Candida albicans* using Molecular Techniques: PCR assay method was used for the detection of the USP indicator pathogens recovered from the tested dosage forms (five *S. aureus* isolates; 1C, 2G, 3N, 17S, 9T, one *Pseudomonas aeruginosa* isolate; 10C and one *Candida albicans* isolate; 9S). This was carried out by using species-specific primer pairs along with other standard reference strains (as positive controls) followed by agarose gel electrophoresis. Each isolate, together with its relevant positive control, produced detectable DNA bands of the expected molecular sizes without non-specific amplification. Furthermore, no amplification was observed in the negative control samples, **Fig. 1**.

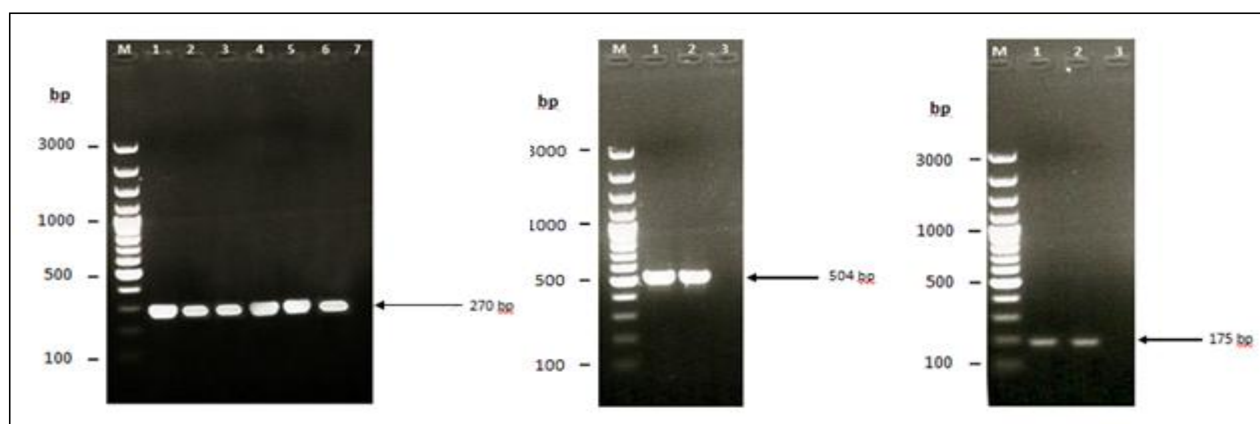

FIG. 1: AGAROSE GELELECTROPHORETOGRAM FOLLOWING PCR AMPLIFICATION OF NUC GENE OF *STAPHYLOCOCCUS AUREUS* ISOLATES (A), OPRL GENE OF *PSEUDOMONAS AERUGINOSA* ISOLATE (B) AND25S RRNA GENE OF *CANDIDA ALBICANS* ISOLATE (C). For (a) Lane M, 100 bp plus DNA ladder; lane 1, *Staphylococcus aureus* ATCC 433001 as a positive control; lane 2, isolate 17S; lane 3, isolate 1C; lane 4, isolate 9T; lane 5, isolate 3N; lane 6, isolate 2G; lane 7, negative control (b) Lane M, 100 bp plus DNA ladder; lane 1, *Pseudomonas aeruginosa* clinical isolate as a positive control; lane 2, isolate 10C; lane 3, negative control (c) Lane M, 100 bp plus DNA ladder; lane 1, Candida albicans clinical isolate as a positive control; lane 2, isolate 9S; lane 3, negative control

Testing of Interferences in PCR Assays by other Organisms: For each tested organism, absence of interference and specificity testing of monoplex PCR assay was carried out using a duplex PCR, consisting of a mixture of the primer pairs of the target gene and the universal primer pairs (in case of bacterial strains). The interference was tested against DNA templates prepared from a panel of different representative Gram-positive and negative strains as well as the indicator organism. Results revealed that a single DG74/RWO1 product (375 bp) was obtained with all bacterial strains, while the species-specific products were obtained only for the corresponding indicator pathogens. Thus, the PCR assay yielded detectable DNA amplicons of expected molecular sizes only in the presence of the respective DNA templates with no additional amplicons for the other co-existing organisms (Supplementary **Fig. S1**).

Detection Limit of PCR Assay for the Purified Genomic DNA of the Specified Tested Organisms: The detection limit of the PCR assay was carried out using ten-fold serial dilutions of the purified genomic DNA (100 ng-1 fg) for each of the indicator pathogens, in separate PCR reactions. The results showed that the lowest amount of template that could be detected was 100 fg for *Pseudomonas aeruginosa* **Fig. 2** and 1 fg for each of *Staphylococcus aureus* and *Candida albicans*.

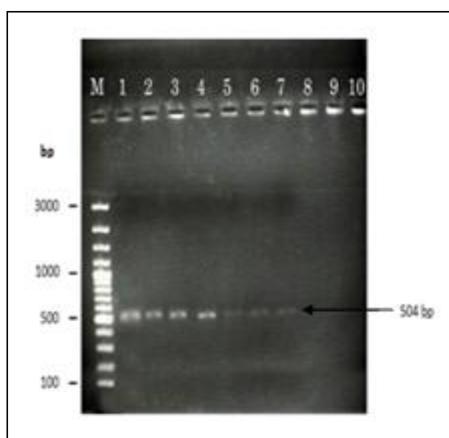

FIG. 2: AGAROSE GEL ELECTROPHORETOGRAM SHOWING THE DETECTION LIMIT OF PCR FOR AMPLIFICATION OF OPRL GENE USING DIFFERENT CONCENTRATIONS (100 NG – 1 FG) OF *PSEUDOMONAS AERUGINOSA* GENOMIC DNA. Lane M, 100 bp plus DNA ladder; lane 1, 100 ng; lane 2, 10 ng; lane 3,1 ng; lane 4, 100 pg; lane 5, 10 pg; lane 6, 1 pg; lane 7, 100 fg; lane 8, 10 fg; lane 9, 1 fg.

Detection Limit of PCR Assay using Whole-Cell Lysates of the Specified Tested Organisms: The detection limit of PCR was evaluated using cell lysates from different dilutions of the test organisms. Results revealed that for all tested organisms, 30 PCR cycles were enough to amplify the target genes from cell lysates of pellets obtained from 1 ml of 10 CFU/ml cell suspensions under the assay conditions. Additionally, the target genes corresponding to *Staphylococcus aureus* could also be amplified from cell lysates of pellets obtained from 1 ml of 10^0 CFU/ml cell suspensions **Fig. 3**, however, at this low cell density (10^0 CFU/ml), the bands corresponding to the amplified target genes appeared rather faint on the gel, lane 5; **Fig. 3**.

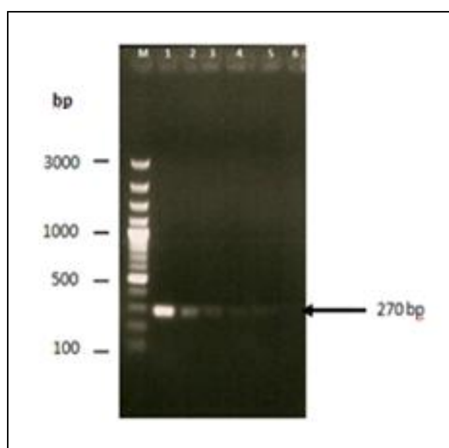

FIG. 3: AGAROSE GEL ELECTROPHORETOGRAM SHOWING THE DETECTION LIMIT OF PCR FOR AMPLIFICATION OF NUC GENE USING CELL LYSATES OBTAINED FROM DIFFERENT DILUTIONS OF S. AUREUS CELLS (10^4 - 10^0 CFU/ML). Lane M, 100 bp plus DNA ladder; lane 1, 10^4 CFU/ml; lane 2, 10^3 CFU/ml; lane 3,10^2 CFU/ml; lane 4, 10^1 CFU/ml; lane 5, 10^0 CFU/ml; lane 6, negative control

Similar limits were obtained for *Candida albicans* (but not for *Pseudomonas aeruginosa*), where the corresponding target genes could be detected at 10° CFU/ml.

Detection Limit of the PCR Assays using Artificially Inoculated Pharmaceutical Preparations with the specified Tested Organisms: The detection limit of PCR was examined for pharmaceutical preparations separately inoculated with appropriate dilutions of the indicator pathogens, to obtain final microbial counts of 10^4 - 10^0 CFU/ml sample. Two prepared cream formulas were separately inoculated with *Pseudomonas aeruginosa* and *Candida albicans,* while a prepared lotion formula was inoculated with *S. aureus* ATCC 433001. The PCR sensitivity was dependent on the DNA extraction method used. Regarding the artificially inoculated lotion, PCR analysis using the gene-jet purification kit showed a minimum detection limit of 10^5 CFU/g (*S. aureus*) at 1:10 dilution of lotion in TSB. This limit improved upon using 1:100 dilution of tested lotion in TSB, where PCR analysis using the Gene-jet purification kit showed a minimum detection limit of 10^0 CFU/g. The boiling-centrifugation technique exhibited lower sensitivity were, at 1:100 dilution of lotion in TSB, a minimum of 10^2 CFU/g sample was the least detected, **Fig. 4**.

In case of the artificially inoculated cream, PCR analysis using the Gene-jet purification kit showed a minimum detection limit of 10^0 CFU/g (*Candida albicans*) and 10 CFU/ml (*Pseudomonas aeruginosa*), at 1:10 dilution of cream in TSB, whilst PCR assays using the boiling-centrifugation technique exhibited lower sensitivity where a minimum of 10^2 CFU/g samples was the least detected.

For both dosage forms, the PCR band corresponding to 10^0 CFU/ml (lane 5) was rather weak, and the result was not reproducible. Sensitivity was improved by the use of double PCR. This was performed by using 10 µl of PCR products from the first 30 cycles as a template for a second PCR round similarly conducted as the first one. The results revealed that by using double PCR, low microbial cell count to a level of 10^0 CFU/ml could be clearly detected with all organisms except *Pseudomonas aeruginosa*.

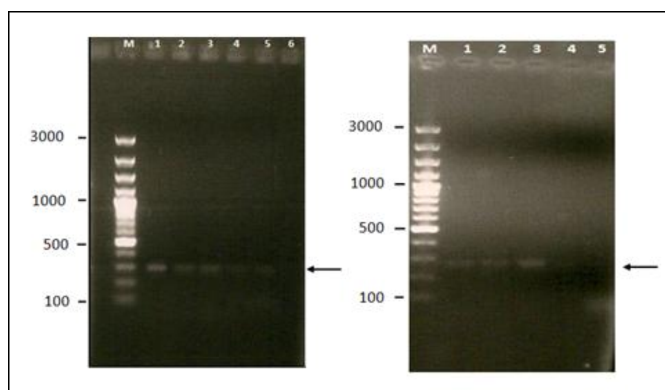

FIG. 4: AGAROSE GEL ELECTROPHORETOGRAM SHOWING THE DETECTION LIMIT OF PCR ASSAY FOR DETECTION OF *STAPHYLOCOCCUS AUREUS* ARTIFICIALLY INOCULATED IN 100-FOLD DILUTED LOTION IN TSB, AT DIFFERENT CELL COUNTS, USING TWO DNA EXTRACTION TECHNIQUES, a) GENE-JET EXTRACTION KIT, b) BOILING-CENTRIFUGATION TECHNIQUE. Lane M, 100 bp plus DNA ladder; lane 1, 10^4 CFU/ml; lane 2, 10^3 CFU/ml; lane 3, 10^2 CFU/ml; lane 4, 10^1 CFU/ml; lane 5, 10^0 CFU/ml; lane 6, negative control

Detection Limit of PCR Assay using Artificially Inoculated Pharmaceutical Preparations with Specified Tested Organisms Together with Other Co-Existing Ones: The minimum reproducible detectable limit in a cream and a lotion preparation, each artificially inoculated with *S. aureus* or *Pseudomonas aeruginosa* together with mixed cultures of *Bacillus licheniformis*, *Staphylococcus epidermidis* and *Enterobacter sawazakii* was 10 CFU/ml for each organism. However, the minimum reproducible detectable limit in a cream and a lotion preparation, each artificially inoculated with Candida albicans together with the three used contaminants, was 10^0 CFU/g.

MPCR for Simultaneous Detection of *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida albicans*: The reaction conditions for the multiplex-PCR assay were optimized to ensure that all the target gene sequences were satisfactorily amplified. The results revealed that the highest intensity for each of the amplified fragments (*Pseudomonas aeruginosa* oprL, *S. aureus* nuc and *Candida albicans* 25S rRNA) was obtained using 0.7 µM of PAL-F/PAL-R primers, 0.4 µM of nuc-F/nuc-R primers, 0.3 µM of CAL4/NL4CAL primers, 3.5 mM $MgCl_2$ and 1X PCR buffer. The reaction profile was 5 min of denaturation at 95 °C and 40 cycles of amplification at 95 °C for 0.5 min, 56 °C for 1.5 min, and 72 °C for 1 min (data not shown).

Using Mixed Cell Cultures of the Three Specified Tested Organisms: The results revealed that mPCR detection was dependent on the DNA extraction method used. MPCR assays using the Gene-jet purification kit for DNA extraction exhibited lower detection limits as compared to mPCR assays using the boiling-centrifugation technique, for the three specified organisms, as shown in **Fig. 5**. The minimum reproducible detectable limit of mPCR assay using the Gene-jet purification kit was 10 CFU/ml, while the minimum reproducible detectable limit using the boiling-centrifugation technique was 10^4 CFU/ml. With the exception of *Pseudomonas aeruginosa*, for mPCR assays using the Gene-jet purification kit, the target genes corresponding to *S. aureus* (nuc) and *Candida albicans* (25S rRNA) could also be simultaneously detected from 10^0 CFU/ml. However, at this low cell density (10^0 CFU/ml), the bands corresponding to the amplified target genes appeared rather faint on the gel (lane 5, **Fig. 5**).

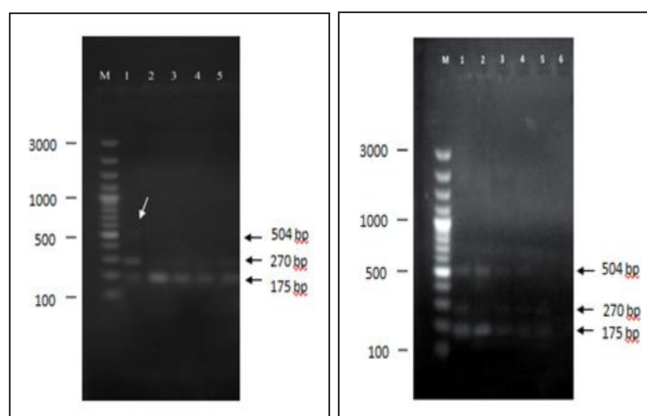

FIG. 5: AGAROSE GEL ELECTROPHORETOGRAM SHOWING THE DETECTION LIMIT OF MULTIPLEX PCR FOR AMPLIFICATION OF *PSEUDOMONAS AERUGINOSA* OPRL GENE, *STAPHYLOCOCCUS AUREUS* NUC GENE AND *CANDIDA ALBICANS* 25S rRNA GENE USING DNA EXTRACTS OBTAINED FROM DIFFERENT DILUTIONS OF PSEUDOMONAS AERUGINOSA/STAPHYLOCOCCUS AUREUS/CANDIDA ALBICANS MIXED CULTURES AND TWO DNA EXTRACTION TECHNIQUES, a) BOILING-CENTRIFUGATION TECHNIQUE b) GENE-JET PURIFICATION KIT. Lane M, 100 bp plus DNA ladder; lane 1, 10^4 CFU/ml; lane 2, 10^3 CFU/ml; lane 3, 10^2 CFU/ml; lane 4, 10^1 CFU/ml; lane 5, 10^0 CFU/ml; lane 6, negative control

Using Artificially Inoculated Pharmaceutical Topical Preparations with the Three Specified Tested Organisms: Likewise, the mixed culture, the PCR detection limit of the artificially inoculated topical preparations with the three tested organisms was dependent on the DNA-extraction method used. Multiplex PCR assays using the

Gene-jet purification kit for DNA extraction showed higher sensitivity compared to mPCR assays using the boiling centrifugation technique. For the artificially inoculated lotion, the minimum detectable limit of mPCR assay for the simultaneous detection of *Pseudomonas aeruginosa, S. aureus,* and *Candida albicans* using boiling-centrifugation technique was 10^2 CFU/g (data not shown), while this limit was 10 CFU/ml in case of Gene-jet purification kit. Additionally, the target genes corresponding to *Candida albicans* and *S. aureus* (but not for *Pseudomonas aeruginosa*), could also be detected at 10^0 CFU/ml, **Fig. 6**. However, at this low cell density (10° CFU/ml), the bands corresponding to the amplified target genes appeared rather faint on the gel, and the result was not reproducible (lane 5, **Fig. 6**).

Similar results were obtained for the artificially inoculated cream where the minimum detectable limit of mPCR assay for the simultaneous detection of *Pseudomonas aeruginosa, S. aureus,* and *Candida albicans* using the Gene-jet purification kit was 10 CFU/ml whereas the minimum detectable limit using the boiling-centrifugation technique was 10^2 CFU/g. The target gene corresponding to *Candida albicans* (but not for *Pseudomonas aeruginosa* nor *S. aureus*), could also be detected at 10° CFU/ml, using the Gene-jet purification kit. However, at this low cell density (10° CFU/ml), this band appeared rather faint on the gel, and the result was not reproducible.

For both tested dosage forms, the detection limit was improved by the use of double PCR. This was performed by using 10 µl of PCR products from the first 30 cycles as a template for a second PCR round that was similarly conducted as the first one. For both dosage forms (cream, lotion), double PCR was attempted on a preparation artificially inoculated to a final count of 10 or 10° CFU/ml with mixed cultures of the three indicator pathogens *Pseudomonas aeruginosa, Staphylococcus aureus* and *Candida albicans*.

The results revealed that for both dosage forms, by using double PCR, target genes corresponding to Staphylococcus aureus and *Candida albicans* (but not for *Pseudomonas aeruginosa*), could be clearly and reproducibly detected at a microbial cell count of 10° CFU/ml.

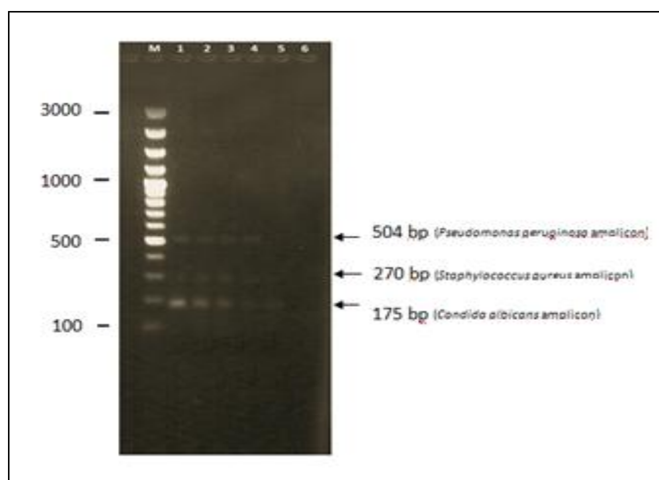

FIG. 6: AGAROSE GEL ELECTROPHORETOGRAM SHOWING THE DETECTION LIMIT OF MULTIPLEX PCR FOR DETECTION OF *PSEUDOMONAS AERUGINOSA, STAPHYLOCOCCUS AUREUS* AND *CANDIDA ALBICANS* WHEN ARTIFICIALLY INOCULATED IN A LOTION PREPARATION, AT DIFFERENT CELL COUNTS (10^4 - 10^0 CFU/ML). Lane M, 100 bp plus DNA ladder; lane 1,10^4 CFU/ml; lane 2, 10^3 CFU/ml; lane 3, 10^2 CFU/ml; lane 4, 10^1 CFU/ml; lane 5, 10^0 CFU/ml; lane 6, negative control

DISCUSSION: On the basis of a significant time reduction to test the pharmaceutical products, different techniques in the field of molecular microbiology are gaining great attention [18, 28, 40-43]. Rapid detection and identification of bacteria, yeast, and mold using PCR technology have been reported in the food industry and clinical laboratories and are also being used in pharmaceutical applications [12, 43]. Simultaneous detection of several microorganisms in a single assay is also possible by using multiplex PCR. In this study, monoplex PCR was developed and was further elevated to multi-plex PCR for simultaneous detection of topical indicator pathogens; *S. aureus*, *Pseudomonas aeruginosa* and *Candida albicans* in pharmaceutical products, in comparison to conventional techniques.

To validate the PCR assay for detecting indicator pathogens against conventional methods, 280 samples were analyzed [32]. The indicator pathogens were isolated by standard methods and biochemically identified from 7 samples in a previous study [32]. Isolation and final identification of the microbial colonies were completed after 5-7 days. Undoubtedly, the use of a simple and rapid detection technique would enhance quality evaluation of pharmaceuticals and consequent consumer protection. In this study, PCR was used for the identification of indicator pathogens in

pharmaceutical samples in a few hours. By recognizing conserved genomic DNA sequences unique to a particular organism and amplifying that region, contamination by that organism can be affirmed. A major effort was directed to determine the applicability of extending the convenience, accuracy and reproducibility of monoplex and multiplex PCR for identification of contaminants in pharmaceutical preparations. As multiplex PCR involves a far more complex reaction system than the normal simplex mode, its performance is more difficult to predict and can be assessed only after several trials [44]. Extensive optimization was thus required in this study to obtain a good balance between amplicons of the various loci being amplified. In agreement with the findings of Henegariu *et al.*, [45] the relative concentrations of the primers were found to be the most important factor in determining equal yields of amplification products from each of tested organisms in a single reaction. Moreover, primers used in this study were selected to have similar annealing temperatures (57, 55 and 58 °C for *Pseudomonas aeruginosa, S. aureus* and *Candida albicans* primers, respectively) and the sizes of amplicons were taken into consideration such that they should not overlap. Other critical factors in mPCR include the concentration of PCR buffer, the balance between $MgCl_2$ and dNTP, the quality of Taq polymerase used and the annealing and extension times [46].

The use of PCR for rapid detection of microbes can be extremely useful for pure microbial cultures, but when applied directly to pharmaceutical samples its efficiency can be markedly reduced [31]. Sample preparation before PCR reaction is a critical step for optimizing any PCR assay [40]. Different DNA extraction methods will affect the PCR analysis differently in terms of specificity and sensitivity. Throughout this study, two DNA extraction procedures were employed; the solid phase absorption method (Gene-jet purification kit) and the boiling-centrifugation method. Our goal was to utilize a sample preparation and DNA extraction method that is simple, rapid, cost-effective and does not need further purification with hazardous and PCR inhibitory substances. Results revealed that even though the boiling-centrifugation method was faster and cheaper than the DNA extraction Kit, the results of the Gene-JET kit showed better efficacy for bacterial DNA extraction from spiked samples and mixed cultures, indicating that it may be more efficient in harvesting bacterial DNA and in reducing the presence of PCR inhibitors than the boiling technique. These findings are in agreement with the findings of Guo [47] where PCR assay using extracted DNA exhibited a sensitivity higher by 1 log when compared to crude DNA.

Moreover, Sepp *et al.*, [48] concluded that the water boiling method may sometimes fail to produce a sufficient quantity and quality of template from samples particularly when the starting material is small and the target molecules are expected to be of low copy number. Despite that, the speed and low cost of the boiling-centrifugation technique must be emphasized.

Additionally, in this study, 1:10 sample dilution was used for all samples tested. At this dilution, none of the tested product types interfered with PCR detection of the indicator pathogens at high counts. However, in lotions, it was not possible to detect indicator bacteria lower than 10^5 CFU/ml, despite using the extraction kit.

This might be attributed to the presence of inhibitors that might have interfered with either the DNA extraction procedure or with the PCR amplification. To overcome this inhibition, a 1:100 sample dilution of lotion was used which resulted in dilution of the inhibitor to a concentration that no longer interfered with DNA extraction or PCR reaction and consequently, PCR amplicons were detected from low template counts. In agreement with this methodology, this level of dilution was used in a similar study for detecting *S. aureus* and *Pseudomonas aeruginosa* in pharmaceutical preparations intended for topical use [49].

Another important aspect to be considered is the sensitivity of PCR assays. Quality of raw materials used for pharmaceutical purposes are satisfactorily controlled, therefore, microbial contamination of pharmaceutical products with a high number of bacteria is not expected. Consequently, the method used for microbiological quality control of pharmaceutical products should be very sensitive. Sensitivity of amplification was dependent on the number of copies of target DNA and was evaluated by use of lysates of whole cells or extracted pure genomic DNA. Sensitivity was tested on both pure cultures and spiked pharmaceutical samples.

The lowest amount of template that could be detected was 1 fg pure DNA and 10^0 CFU/ml pure culture for S.aureus and Candida albicans and 100 fg pure DNA and 10 CFU/ml pure culture for *Pseudomonas aeruginosa*. Published observations in which a detection sensitivity of 0.69 pg of purified genomic DNA and 6 CFU of *S. aureus* cells [37] and 100 CFU/ml of *Pseudomonas aeruginosa* cells [36], were reported. When applied on artificially inoculated pharmaceutical samples, the minimum reproducible detectable limit for each of the indicator pathogens was 10 CFU/ml. However, for *S. aureus* and *Candida albicans*, dilutions corresponding to lower cell numbers, 10^0 CFU/ml, yielded a band inconsistently, probably because of stochastic variations in the actual numbers of cells present in the volume of the dilution sampled. The sensitivity was improved by the use of double PCR, where after two consecutive identical PCR rounds, as low as 10^0 CFU/ml of *Candida albicans* and *S. aureus*, could be reproducibly detected. The sensitivity in this regard was superior to those reported in previously published observations where adapted PCR assays were reported to detect 1-10 CFU of bacteria per gram or milliliter of pharmaceutical product however only after 24 h pre-enrichment [18, 40]. The PCR assays conducted in this study, was proven to show high sensitivity and specificity not only in detecting indicator pathogens when found as single contaminants in pharmaceutical preparations but also in detecting indicator pathogens in pharmaceutical preparations in the presence of other potentially contaminating bacteria.

One of the problems often encountered with mPCR is a reduction in sensitivity. Many authors have reported a 10 fold reduction in sensitivity of mPCR when compared with monoplex PCR [50-51] and attributed this reduction to the competition between individual reactions for dNTPs and Taq polymerase when multiple primer sets are combined in a single reaction [52]. However, in this study, we were able to demonstrate that by optimising the reaction conditions and template concentrations, the sensitivity level of the mPCR was comparable to that of monoplex PCR and that no reduction in sensitivity was experienced. However, PCR products of the indicator pathogens' in the multiplex PCR were poorer by visualization on agarose gels than that in the monoplex PCR.

The minimum detectable limit of mPCR assay for the simultaneous detection of *Pseudomonas aeruginosa*, *S. aureus* and *Candida albicans* in mixed cultures and pharmaceutical preparations was 10 CFU/ml. Similar results were observed by Farajnia *et al.*, [30] where less than 10 CFU/ml was detected using a mPCR assay for detecting indicator pathogens. In this study however, the target genes corresponding to *Candida albicans* and *S. aureus*, could also be detected at 10^0 CFU/ml, however, at this low cell density (10^0 CFU/ml), the bands appeared rather faint on the gel and the result was not reproducible. The sensitivity was improved by the use of double PCR, where after two consecutive identical PCR rounds, as low as 1 CFU/ml of *Candida albicans* and *S. aureus*, could be easily and reproducibly detected. Published observation in which the detection sensitivity was improved following double PCR was reported [53].

Detection of microbial indicators in pharmaceutical samples using PCR were previously described by several authors [18, 26, 28, 29, 31, 54, 55]. However, a culture pre-enrichment step was included to increase the target bacterial concentrations before they were detectable by PCR. Moreover, Jimenez showed simultaneous detection of *E. coli, S. aureus, P. aeruginosa*, and *A. niger*, in pharmaceutical samples with detection limits of 10 CFU/ml, using RoboCycler 96-gradient PCR which utilized a gradient profile that allowed the use of primers with different annealing temperatures [56]. In the current study, however primers were chosen such that they anneal at single temperature (56 °C) and the extraction procedures and reaction volumes used enabled the detection of low levels of contaminants without prior enrichment. This was achieved by preparing more concentrated DNA extracts through a reduction in the volume of the elution buffer used (30 µl) and by incorporating higher volumes of DNA template in subsequent PCR reactions (10 µl). This resulted in an increase in the number of target cells per PCR reaction, thus attaining higher sensitivity while obviating the need for sample pre-enrichment. Furthermore, none of the published studies included the detection of *Candida albicans*, which is an important fungal indicator specified in the harmonized Pharmacopoeias.

When artificially contaminated samples were simultaneously analyzed by PCR assays and standard microbiological procedures, there was a 100% correlation between the two methods.

However, PCR detection of microbial contamination required about 8 h while standard microbiological methods were completed within 5-7 days. Compared with the monoplex PCR assay that detects only a single pathogen, the multiplex assay detected the three target indicator pathogens from pharmaceutical samples in one reaction tube therefore simplifying the entire detection process and eliminating the need for multiple differential and/or selective media. By reducing the time, work and materials needed to detect pharmaceutical pathogens, better monitoring of pharmaceutical safety is available. However, unfortunately conventional PCR assays do not provide viability data as they cannot differentiate between living and dead cells; this might be one of the reasons why standard microbiological techniques are still favored over PCR for quality control purposes.

Moreover, despite the presence of real-time technologies for detection and quantification of pathogens, yet the cost of instrumentation and consumables of these technologies limit their use in the pharmaceutical industry. Quantitation is not significant in this scope of study since the pharmacopoeia necessitates the absence of microbial indicators in 1 gm sample product which is what we demonstrated in this study using simple, user-friendly, feasible PCR technology. The benefits of real-time technologies are less likely to be found in the pharmaceutical laboratory and more likely to be seen by the clinician or the health care administrator [57].

Thus, the present assay requires moderate level training and aims to detect microbial contaminants providing visible results quickly and accurately. Using relatively inexpensive materials and equipment, a quality control industrial laboratory would be able to incorporate this procedure and run diagnostic assays on a large scale basis to detect pharmaceutical contaminants [35]. The implications of the present study are promising and the level of sensitivity achieved is applicable to the practical survey of microbial contamination in pharmaceutical samples [31].

CONCLUSION: PCR analysis provides rapid quality evaluation of pharmaceuticals and can thus be used as an alternative to the lengthy cumbersome conventional isolation and identification procedures for exclusion of contamination by certain viable indicator microorganisms. The technique has also been proven to be as accurate and less costly than traditional identification techniques. Even in case of contamination by non-viable indicator organism, PCR technique can still be used for detection after partial incubation of cultivated test sample. Thus, the application of PCR by pharmaceutical companies will allow rapid implementation of corrective actions resulting in the minimization of manufacturing losses, significant cost savings, and optimization of resources and risk assessment.

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