Using Uniplex and Multiplex Polymerase Chain Reaction Assays For Molecular Diagnosis Of Indicator Pathogens in Non Sterile Pharmaceutical Products

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Abstract: Detection of four USP indicator bacteria (*Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhymurium*) is one of the mandatory tests in microbial quality of non sterile pharmaceutical products; therefore, rapid and sensitive detection of the contaminations is of great importance for product release. Thus, the aim of this study was to develop a multiplex PCR (mPCR) assay for simultaneous detection and identification of four indicator pathogenic bacteria in a single reaction. Uniplex PCR was performed for the detection of each microorganism individually targeting the conserved region in each bacterial genome. A multiplex PCR was used as an alternative rapid & accurate technique for detection of these indicator bacteria in samples of oral and topical products. Specific primers for indicator bacteria, were applied to allow simultaneous detection of them, and the sensitivity and specificity of each primer pairs were determined. Validation of the PCR analysis scheme was performed for every product, to exclude the inhibitory and masking effect of the product formula on the contaminated microorganisms. In the mPCR with mixed DNA samples, specific bands for corresponding bacteria were simultaneously detected. Agarose gel electrophoresis of PCR products revealed 100% specificity of mPCR with single bands in the expected sizes. Low levels of microbial contamination less than 10 cfu per milliliter or gram of product were detected using mPCR assay. Gel electrophoresis results showed that all tested samples, which were inoculated artificially with the control bacteria, were free of interfering substances. The detection of all four indicator pathogenic bacteria were completed in less than 24 h with this novel mPCR method, whereas the conventional USP methods and uniplex PCR required 5–6 days and 27 h for completion, respectively. It was concluded that the application of mPCR technology in microbial quality control of non sterile pharmaceutical products can be performed in rapid and accurate detection of objectionable microorganisms and allows for the cost-effective detection of all bacterial pathogens and timely manner in pharmaceutical industry, which leads to faster release of products and more rapid implementation of corrective actions.

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1. Introduction

Conventional methods of detecting bacteria in pharmaceutical contaminants comprise propagation in selective enrichment media followed by confirmation and biochemical tests. These methods are very laborious and time consuming and not always specific enough to discriminate among species and strains (Denyer*et al*., 2004). However, Standard microbiological analysis of pharmaceutical samples requires 5–7 days to be completed (Hefni, 1987**;** Palmieri*et al.,* 1988 and Casey *et al*., 1998, 2002). 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. Rapid methods provide reliable and cost-effective analysis for the microbiological evaluation of pharmaceutical environments. Molecular methodologies such as Adenosine triphosphate (ATP) bioluminescence and PCR -based assays provide rapid quality control analysis of cosmetic and pharmaceutical finished products and raw materials. Rapid release of samples has resulted in the optimization of manufacturing, product testing and release allowing high throughput and simultaneous analysis of pharmaceutical formulations. Rapid methods have given results within 24 to 30 hours (Ignar *et al*., 1998; Jimenez, *et al.,*1998). Therefore, for microbiological monitoring of products and raw materials, there is a demand to develop and apply new technologies which will be rapid, sensitive, accurate and cost-effective (Van Der Zee, *et al.,*1997). The use of rapid technologies for quality control evaluation of finished products and raw materials has resulted in optimization of product release and manufacturing (Jimenez, 1998). However, new molecular methods are available that can rapidly detect microorganisms in contaminated samples. PCR amplifies genetic sequence of the microbial contaminant (Ignar*et al*., 1998) without compromising specificity and sensitivity.

Jimenez *et al.,*(1999) developed and compared PCR assays to standard methods for rapid

detection of the USP bacterial indicators i.e. *E. coli, S. aureus& P. aeruginosa,* in artificially contaminated pharmaceutical raw materials and finished products with more than 10 cfu in 2% ($^{w}/_{v}$) product. Samples were incubated for 24hr at 35°C in lactose broth &Trypticase soy broth, and then streaked into selective growth media & 4 ml lysate aliquote for bacterial DNA detection using PCR. After 5-6 days, all microbial indicators were morphologically & biochemically identified using standard methods while detection using PCR was completed in 27-30hr. Jimenez *et al.* (2000) developed another PCR assays for quality evaluation of pharmaceutical raw materials and finished products with low levels of microbial contamination. Samples were artificially contaminated with less than 10 CFU of *E. coli, S. aureus, P. aeruginosa,* and *Aspergillusniger*. Bacterial DNA was extracted from each enrichment broth by mild lysis in Tris-EDTA-Tween 20 buffer containing proteinase K while mold DNA was extracted by boiling samples in Tris-EDTA-SDS buffer for 1 h. A 10- pi aliquot of extracted DMA was added to Ready-To-Go PCR beads and specific primers for *E. coli, S. aureus, and P. aeruginosa.* However, 50- ul aliquots of extracted mold DMA were used for amplification of. specific*A. niger*DMA sequences.

Merker*et al.* (2000), employed Fluorescence-coupled PCR technology to quantify DNA segments specific for *S. aureus, P. aeruginosa, and Enterobacteriaceae.* The PCR procedure is put forward as an alternative method for detecting microbial contaminations in pharmaceutical preparations and is compared to the tests for specified microorganisms described in European Pharmacopoeia (EP) and the USP. methods.

However, Jimenez *et al.* (2001) evaluated the same system for detection of the presence of *S. typhimurium*in artificially contaminated samples of raw materials ,cosmetic and pharmaceutical products with mixed bacterial cultures of *E. coli, S. aureus& P. aeruginosa.* Samples were preenriched in lactose broth, then analysed using PCR & standard methods. The BAX (™) system allowed a faster quality control evaluation of those raw materials and cosmetic/pharmaceutical formulations that require *Salmonella* spp. screening. Rapid quality evaluation of pharmaceutical samples resulted in optimization of product manufacturing, quality control, and release of finished products. For this purpose, rapid molecular methodologies were developed for rapid quantitative & qualitative information on microorganisms present in a given pharmaceutical samples. However, validation & implementation of these new methods are not widely adopted by industry. This is due to: absence of validation guidelines, uncertain regulatory status, lack of validation & technical support, lack of understanding of technologies, hesitation from managers & companies to try new methods, lack of resources for technology evaluation & implementation, systems are expensive & underestimation by a vendors of regulatory requirements (Jimenez , 2004a).

Multiplex PCR assays have been developed and validated for environmental, food and clinical analyses (Knabbel and Crawford, 1995; Brasher *et al.,* 1998). However, multiplex PCR assay has been developed for the detection and identification of indicator bacteria in pharmaceutical finished products of ophthalmic, chemotherapeutic, psychiatric, cardiac and gastrointestinal drugs.Multiplex PCR assay provides sensitive and reliable results and allows for the cost-effective detection of all four bacterial pathogens in single reaction tube (Karanam*et al*., 2008 and Farajina*et al*., 2009). The aim of this study was to develop mPCR assay for simultaneous detection and identification of four indicator pathogenic bacteria in a single reaction, as well as the present study was undertaken toshow the tested samples, which were inoculated artificially with the control bacteria, were free of interfering substances.

2. Materials and methods

A. Materials

I. Specimens:

Samples of commercially available finished pharmaceutical products were tested. These sample representing fifty pharmaceutical samples were tested using mPCR; where twelve multivitamin syrup samples, sixteen antihistaminic tablet samples and twelve capsule samples in addition to four antirheumatic cream samples for topical and six analgesic stored samples. five non-sterile pharmaceutical dosage forms were collected from different Saudi manufacturers. All of them were approved by the Saudi Ministry of Health in accordance with the monographs specified in pharmacopoeias (USP, SP, EP and BP).

II. Bacterial strains and growth conditions:

The standard strains of *E. coli* (ATCC 11775), *E. coli* (ATCC 25922), *E. coli* (ATCC 8739), *E. coli* (ATCC 8745), *S. aureus* (ATCC 25923), *S. aureus* (ATCC 6538), *S. aureus* (ATCC 12600), *S. typhimurium* (ATCC 6994), *S. typhimurium* (ATCC 14028), *S. typhi* (ATCC 19430), *S. paratyphi* (ATCC 9150), *Ps. aeruginosa* (ATCC 27853), *Ps. aeruginosa* (ATCC 9027), *Ps. aeruginosa* (ATCC 10145), were included in validation of the microbial limit tests, challenge test and PCR study. All were from American Type Culture Collection (ATCC), purchased from remelculti-loops which were ready to use, disposable inoculating loops containing

stabilized, preserved and viable microorganisms. The loop was dissolved in rehydration fluid or streaked directly onto an appropriate media. Pure cultures were maintained on slants at 4°C for working purposes, and stock cultures were stored in 15% glycerol at -70°C.

III. Reagents

1.0.5 M EDTA (pH 8.0):

Add 186.1 g of disodium ethylene diamine tetraacetate. $2H_2O$ (Sigma) to 800 ml of d.H_20. Stir vigorously on a magnetic stirrer. Adjust the pH to 8.0 with NaOH (-20 g of NaOH pellets). Dispense into aliquots and sterilize by autoclaving.

2.1 M Tris-base:

Dissolve 121.1 g of Tris base (Sigma) in 800 ml of H_2O. Adjust the pH to the desired value by adding concentrated HC1.

pHHC1
7.6 60 ml.
8.0 42 ml.

Allow the solution to cool to room temperature before making final adjustments to the pH. Adjust the volume of the solution to 1 liter with H_2O. Dispense into aliquots and sterilize by autoclaving.

3. TE Buffer (pH 7.6):

1 MTris-base (pH 7.6) 10.0 ml.
0.5MEDTA(Ph 8.0) 2.0 ml.
Sterile d.H_20 988 ml.

4. 10% Sodium Dodecyl Sulfate (SDS):

Dissolve 100 g of electrophoresis-grade SDS (Sigma) in 900 of d.H_2O. Heat to 68°C to assist dissolution. Adjust the pH to 7.2 by adding a few drops of concentrated HC1. Adjust the volume to 1 liter with H_2O. Dispense into aliquots.

5. Proteinase K (20 mg/ml):

Dilute 100 mg of proteinase K in 5 ml sterile d.H_2O and aliquots in 10 tubes. Store at -20°C.

6. Phenol/Chloroform/Isoamyl alcohol (25:24:1) (Sigma).

7. 3M Sodium Acetate (pH 4.8 and pH 5.7):

Dissolve 408.1 g of sodium acetate. $3H_2O$ (Sigma) in 800 ml of distilled.H_2O. Adjust the pH with glacial acetic acid. Adjust the volume to 1 liter with H_2O. Dispense into aliquots and sterilize by autoclaving.

8. 100% Ethanol (Fisher Scientific, Bohemia, N.Y, U.S.A.).

9. 70% Ethanol.

10. Ribonuclease A:

Dissolve pancreatic RNAase (Sigma) at a concentration I mg/ml in TE buffer. Heat to 100°C for 15 minutes. Allow to cool to room temperature. Dispense into aliquots and store at -20°C.

11. Electrophoresis Buffer (Tris-borate buffer) (TBE):

A. l0x Stock Solution, 1 liter:

Tris-base (Sigma) 108 gm.
Boric acid (Sigma) 55.0 gm.
0.5 MEDTA, Ph 8.0 40.0 ml.

Sterilize by autoclaving for 20 minutes at 15 Ib/sq. in. on liquid cycle and store at room temperature.

B. Working Solution (Ix TBE):

10x TBE 100 ml.
Sterile d.H_2O 900 ml.

12. Agarose Gel (1.5%):

Electrophoresis-grade agarose powder (GIBCO Bethesda Research Lab.; Life Technologies, Grand Island, N.Y., U.S.A.) was added to Ix TBE gel buffer and melted by boiling for several minutes. Be sure all agarose particles were completely melted.

13. Gel Loading Buffer (Tracking Dye):

Bromophenol blue (Sigma) 0.25 gm.
Sucrose (GenAR) 40.0 gm.
Sterile d.H_2O 100 ml.
Keep at 4°C until use.

14. DNA Marker: 1 kb DNA molecular weight marker (GIBCO-BRL).

15. Ethidium Bromide Solution (10 mg/ml):

Add 1 g of ethidium bromide (Sigma) to 100 ml of sterile d.H_20. Stir on a magnetic stirrer for several hours to ensure that the dye has dissolved. Wrap the container in aluminum foil and store at room temperature.

Caution: Ethidium bromide was a powerful mutagen and was moderately toxic. Gloves should be wear when working with solutions that contain this dye.

16. GeneAmp PCR Core Reagents Kit (Perkin Elmer,Norwalk, CT, USA).

17. Oligonucleotide PCR Primers(Perkin Elmer-Applied Biosystem Inc., Foster City, Calif., USA).

18. PCR Purification Kit (Qiagen Inc.)

19. Base Sequences of Oligonucleotide Primers as shown in table 1.

Table(1): List of bacterial target, specific gene and base sequences of oligonucleotide primers with PCR product size.

Bacterial target	Target gene	Primer sequence	PCR product (bp)
Salmonella spp.	*Inv A*	5′ATCGCCACGTTCGGGCAATTC3′, 5′ACGGTTCCTTTGACGGTGCGAT3′	275
Staphylococcus aureus	*m-RNA nuclease*	5′TTCGAAAGGGCAATACGCAAAGA3′, 5′GCTTTAGTTCGTCAAGGCTTGGCTA3′	461
Pseudomonas aeruginosa	*oprL*	5′CGGGCGTGCTGATGCTCGTAT3′, 5′GCGCGAGGAACGTCAGGACAC3′	709
Escherichia coli	*16S rRNA*	5′CCGTGTTCTCATCCTCCCGCCTC3′, 5′TCTCCGGTGGTCAGCGTCAGCGT3′	559

B-Methods

Conventional analysis (Hefni; 1987)

The pharmaceutical samples were inoculated in trypticase soy broth and fluid lactose broth. These suspensions were incubated at 35°C for 24–48 h for enrichment. After incubation, the enriched broths were streaked onto selective/differential agar for isolation of the target microorganisms. After incubation, representative bacterial colonies were selected based on their morphological characteristics. *E. coli* shown as green metallic sheen colonies on EMB agar, and lactose fermenting non-mucoid colonies on MacConkey agar.*Salmonella* shown as red colonies with or without black centers on XLD agar and reddish pink colonies on BGA.*P. aeruginosa*shown as greenish fluorescence colonies on cetrimide agar.*S. aureus*shown as shiny black colonies with opaque zone and fermented colonies on mannitol–salt agar. These colonies were further confirmed by biochemical and serological analysis.

Pharmacopoeial Methods (USP; 2000; 2006; 2007)

The artificially inoculated test samples with four indicator bacteria diluted 10 times with phosphate buffer pH 7.2 were streaked onto selective/ differential agars for morphological and biochemical identification. In the following step, a serial dilution of each specific bacterium was prepared and 1 mL of the resulting solutions was subjected for colony counting by pour plate method. The results demonstrated the minimum detectable amount of indicator bacteria in the test samples by routine USP standard procedure: *E. coli*, 18 cfu/mL; *S. aureus*, 22 cfu/mL; *P. aeruginosa*, 25 cfu/mL; and *S. typhimurium*, 20 cfu/mL in a time period of 5–6 days.

Detection of bacterial pathogens using polymerase chain reaction:

The validity of the pharmacopoeia test results was largely based on a demonstration that the examined product does not inhibit the multiplication of the microorganisms that may be present. Therefore, a preparatory test using four standard strains, including *S. aureus* (ATCC 6538), *P. aeruginosa* (ATCC 9027), *E. coli* (ATCC 8739) and *S. typhymurium* (ATCC 14028), was conducted according to the United States Pharmacopeia (USP 2007). In the next step, samples of pharmaceutical products which were used as a model of non-sterile pharmaceutical product, diluted 10 times with sterile phosphate buffer pH 7.2 (10mL), were artificially inoculated with a serial dilution of four bacteria called indicator potential pathogens and other bacteria as a control in the concentration range of 100 cfu/mL. The concentration range of indicator bacteria was prepared using serial dilution of a stock of each bacterial inoculums in a sterile buffer. One milliliter of each dilution was used for the enumeration through the pour plate technique and simultaneously the other equal volumes were subjected to the USP standard procedure of identification, as well as PCR method.

Ten gram of samples were enriched in 100 ml of trypticase soy broth containing 4% Tween-20 and 0.5% soy lecithin for *E. coli, S. aureus* and *P. aeruginosa*, and 10 g in 100 ml of buffered peptone water (BPW) for *S.typhymurium*. And incubated at 37 °C for a period of 16 h. Exactly 10 ml of enriched culture were centrifuged at 12,000 rpm for 5 min at 4 °C from all the samples and the pellet was used for DNA extraction. For standard conventional analysis, the USP procedure was followed for the detection of *E. coli, S.typhymurium.,S. aureus* and *P. aeruginosa*. Inoculated samples were incubated at 35°C. Fluid lactose broth enrichments were streaked on eosine methylene blue agar (EMB), MacConkey agar, xylose lysine deoxycholate agar (XLD) and brilliant green agar (BGA). Trypticase soy broth enrichments were streaked on cetrimide agar, Baired Parker agar (BPA) and mannitol–salt agar. After 24–48 h of incubation at 35 °C, colonies were streaked onto sterile plates of trypticase soy agar (TSA) for isolation of pure cultures. TSA plates were incubated for 18–24 h and cells from pure cultures were gram stained and further biochemical identification of the bacteria was performed using coagulase and oxidase test for *S. aureus and P. aeruginosa*, respectively

(USP, 2006). All the experiments were performed in triplicates.

a) Pretreatment of the samples:

1. Tablets were grinded in a sterile mortar

3. Fatty water insoluble products were homogenized aseptically with 5gm polysorbate 80 (emulsifying agent) or heated to not more than 40° C, & mixed carefully while maintaining the temperature in water bath for shortest time.

b) Sample preenrichment:

1. Test for *S. aureus, E. coli&S. typhimurium* in oral samples: (Jimenez *et al.*, 2001)

Ten grams of pretreated samples were added aseptically into two 500ml of buffered peptone water (BPW) containing 4% polysorbate 20, to avoid the masking effect of bacterial flora in samples on *S. typhimurium.* To test the validity of the method one of the pretreated samples in BPW was inoculated with *S. aureus, E. coli&Salmonella* by adding 1 ml of the 100 CFU/ml culture prepared. Samples flasks were incubated for 24 hours at 35°C then 1 ml of preenriched samples was subcultured into 9 ml of Brain Heart Infusion (BHI) broth followed by incubation at 35°C for 3 hours.

2. Test for *S. aureus, P. aeruginosa* in topical samples (Jimenez *et al*., 1999)

Ten grams of pretreated samples were added aseptically into two 100ml of TSB containing 4% polysorbate 20. To test the validity of the method one of the pretreated samples in TSB was inoculated with *S. aureus& P. aeruginosa* by adding 1 ml of the 100 CFU/ml culture prepared. Samples flasks were incubated for 24 hours at 35°C.

c. DNA Extraction (Jimenez *et al.*, 1999 and Farajnia*et al*., 2009):

1. Five milliliter from pharmaceutical products diluted 1:10 in pH 7.2 phosphate buffer was deliberately contaminated with a serial dilution of *Salmonella spp., S. aureus, E. coli* and *P. aeruginosa.*

2. Grow in shaker water bath (200 rpm) at 37°C for 6 h in an orbital shaker.

3. Spin 1.5 ml of the culture in a microcentrifuge at 6000 rpm for 2 min. discard the supernatant.

4. Resuspend pellet in 500 ul TE (pH 7.6) buffer by repeated pipetting. Add 30 ul of 10% SDS and 3 ul of 20 mg/ml proteinase K to give a final concentration of 100 ug/ml proteinase K. Mix thoroughly and incubate 1 hr at 37°C.

5. Add an equal volume of phenol/chloroform/isoamyl alcohol (25:24: 1) to the DNA solution to be purified.

6. Vortex vigorously for 10 sec.

7. Spin for 5 min in a microcentrifuge at 8000 rpm.

8. Carefully remove the top (aqueous) phase containing the DNA using a 200-ul pipette and transfer to a new tube.

9. If a white precipitate was present at the aqueous/organic interface, repeat steps 4 to 7.

10. Add 1/10 volume of 3 M sodium acetate, pH 5.7, to the solution of DNA. Mix by flicking the tube several times with a finger.

11. Add 2 volume of ice-cold 100% ethanol. Mix by flicking the tube several times with a finger and place in a -70°C freezer for at least 20 min.

12. Spin for 10 min in a microcentrifuge at 9000 rpm and pour off the ethanol supernatant

13. Add equal volume of 70% ethanol. Invert the tube several times and spin for 5 min in microcentrifuge as before.

14. Pour off the supernatant as before and allow the DNA to air dry.

15. Resuspend the pellet in 100 ul TE buffer pH 7.6 containing DNAase-free pancreatic RNAase.

16. Determine the concentration of DNA by spectrophotometry; take O.D. reading at wavelengths of 260 nm and 280 nm. The reading at 260 nm allows calculation of the concentration of nucleic acid in the sample. An O.D. 260 of 1 corresponds to approximately 50 ug /ml for double-stranded DNA, 40 ug/ ml for single-stranded DNA. The ratio between the readings at 260 and 280 nm provides an estimate of the purity of the nucleic acid. Pure preparations of DNA have O.D. 260 / O.D. 280 values of 1.8.

17. The pure DNA was subjected to uniplex and multiplex PCR assays.

d. Primer determination and synthesis:

The primer sequences utilized for detection of the four selected bacterial pathogens were shown in **table 1.** The primers were synthesized on a Model 380B DNA synthesizer (Perkin Elmer-Applied Biosystem Inc.). Before use, the primers were desalted through a Sephadex G-25 column (Pharmacia Inc., Piscataway, N.J., U.S.A.). The concentration of the primers was measured by spectrophotometry, and the primers were aliquoted in 50-ul volumes and stored at -20°C.

e. Optimization of uniplex PCR assay:

Individual PCR reactions for *E.coli*, *S. typhimurium.*,*S.aureus* and *P.aeruginosa* were optimized using reagents from GeneAmp PCR Kit (Perkin Elmer). A reaction volume of 100 ul of PCR mixture contained 100 mMTris-HCl (pH 8.3); 500 mM KC1; 200 uM each of dATP, dCTP, dGTP, and dTTP and 1 U AmpliTaq DNA polymerase. Optimization of the individual PCR assays were done with different concentrations of magnesium chloride (1.5 and 2.5 mM) and primer (1.0, 2.5, 5.0, 7.5, and 10.0 pmol). Different concentrations of DNA (25,

12.5, 6.25, 3.12, 1.56, 0.78 and 0.39 ng) were then added to the mixture. The reaction mixture was overlaid with 50 ul of mineral oil. PCR was performed in an automatic DNA thermal cycler. Following preliminary trials with different annealing temperatures and times. The positive control consisted of DNA isolated from each standard bacterial strain grown in enrichment broth. The negative controls included PCR mixtures with primers, but without DNA. The thermal cycler was programmed for optimum PCR conditions. Initially, the reaction mixture was heated at 95°C for 5 minutes. Then the PCR was run for 35 cycles at a melting temperature of 95°C for 30 s, annealing temperature of 55°C for 30 s, and extension temperature of 68°C for 1 min. The sample was then heated at 68°C for 7 minutes for the final extension reaction.

f. Preparation of the horizontal agarose gel electrophoresis:

1. Prepare an adequate volume of electrophoresis TE buffer to fill the electrophoresis tank and prepare the gel.
2. Add the desired amount of electrophoresis-grade agarose to a volume of electrophoresis buffer sufficient for constructing the gel. Melt the agarose in a microwave oven and swirl to ensure even mixing. Gels typically contain 1.5% agarose.
3. Seal the gel casting platform by taping the open ends with adhesive tape. Pour in the melted agarose and insert the gel comb, making sure that no bubbles are trapped underneath the comb and all bubbles on the surface of the agarose are removed before the gel sets.
4. After the gel has hardened, remove the tape from the open ends of the gel platform and withdraw the gel comb, taking care not to tear the sample wells.
5. Place the gel casting platform containing the set gel in the electrophoresis tank. Add sufficient electrophoresis buffer to cover the gel to a depth of about 1 mm (or just until the tops of the wells were submerged). Make sure no air pockets were trapped within the wells.
6. DNA samples should be prepared in a volume that was not overflow the gel wells by addition of the appropriate amount of gel loading buffer. Samples were typically loaded into the wells with a Micropipette. Be sure to include DNA molecular weight marker.
7. Be sure that the leads were attached so that the DNA was migrate into the gel toward the anode or positive lead. Set the voltage to the desired level, typically 80 V, to begin electrophoresis. The progress of the separation can be monitored by the migration of the dyes in the loading buffer.
8. Turn off the power supply when the Bromophenol Blue dye from the loading buffer has migrated a distance judged sufficient for separation of the DNA fragments.

g. Detection of PCR products:

Examine the reaction products on a gel electrophoresis . A volume of 15ul of amplified PCR products was subjected to electrophoresis at 80 V in horizontal gels containing 1.5% agasrose with Tris-borate buffer. The gel was stained with ethidium bromide, exposed to UV light to visualize the amplified products and photographed. 1 kb DNA molecular weight marker was used for determining the size of the amplified fragments.

h. Optimization of multiplex PCR (mPCR) assay:

The optimal conditions for mPCR assay were applied by varyingconcentrations of MgCl2 (1.5 and 2.5 mM), Taq DNA polymerase (1 U and 2 U per reaction) and primers (1.0, 2.5, 5.0, 7.5, and 10.0 pmol). The positive control consisted of DNA isolated from all four bacterial strains grown in a broth culture. The negative controls includes PCR reaction mixture with all four primers, but withoutDNA. Reactions were carried out in an automated thermal cycler with an initial denaturation at 95 °C for 5 min followed by 35 cycles at 95, 55, and 68 °C for 30 s, 30 s, and 1 min, respectively, and a final extension at 68 °C for 7 min.

i. Specificity of multiplex PCR:

The PCR primers were also examined for their specificity. Reactions were carried out in an DNA thermal cycler with an initial denaturation at 95 °C for 5 min, followed by 35 cycles at 95, 55, and 68 °C for 30 s, 30 s, and 1 min, respectively, and a final extension at 68 °C for 7 min.

j. Sensitivity of multiplex PCR on chromosomal DNA:

The sensitivity of multiplex PCR was also examined by varying DNA concentrations ranging from (25, 12.5, 6.25, 3.12, 1.56, 0.78 and 0.39 ng), and primers (1.0, 2.5, 5.0, 7.5, and 10.0 pmol). The reactions were carried out in an automated DNA thermal cycler with an initial denaturation at 95 °C for 5 min, followed by 35 cycles at 95, 55, and 68 °C for 30 s, 30 s, and 1 min, respectively, and a final extension at 68 °C for 7 min.

k. Sensitivity of multiplex PCR on bacterial cultures:

A total of 10 artificially contaminated pharmaceutical samples were analyzed. These samples were inoculated separately into pre-enrichment broths with 10% (w/v) of product. Among 10 samples, 5 samples were inoculated with 20 CFU/ml and remaining 5 samples were inoculated with 10 CFU/ml. Bacteria were lysed by mixing the samples with an equal volume of 0.6 MNaCl, 0.2

MNaOH, 0.1% sodium dodecyl sulfate. The mixture was incubated for 5 min at room temperature and used immediately to identify the pathogens in uniplex and multiplex PCR methods **(Jones and Mobley, 1987).**

3. Results

Detection of indicator pathogens in artificially inoculated pharmaceutical products using Uniplex PCR assay

Uniplex PCR was employed to detect four bacterial pathogens, *Ps. aeruginosa, E. coli, S. typhimurium*and *S. aureus*from different pharmaceutical samples. The primer sequences, target region, and amplicon sizes were summarized in table (2).

The results revealed that the primers were very sensitive and specific to detect bacterial pathogens by adopting annealing temperature at 55 °C, 10 ng of DNA, 5 pmol of primer and 1.5 mM $MgCl_2$ concentration. The amplified bacterial pathogens were detected by gel electrophoresis and staining with ethidium bromide then examining it under UV light. Since, uniplex PCR depicted promising results, and for the reason that all pathogens were detected at similar annealing temperature (55° C), we devised multiplex PCR. (Fig.1).

Detection of indicator pathogens in artificially inoculated pharmaceutical products using multiplex PCR assay

The multiplex PCR assay was optimized by varying the primer and $MgCl_2$ concentrations. But bands were distinct when species-specific primers were 2.5 pmol of each primer/reaction, 1.5 mM $MgCl_2$, and annealing at temperature 55 °C. The same conditions were adopted for further studies. The multiplex PCR results determined the presence of four bacterial pathogens in single reaction with varying amplicon sizes *P. aeruginosa*(709 bp)*; E. coli* (559 bp)*; S. aureus*(461 bp); *Salmonella* spp. (275 bp), respectively, as shown in Fig. 2.

To ensure the specificity of the primers used in above multiplex PCR assay, primers were cross-examined with pharma contaminants with varying primer concentrations. Multiplex PCR was carried out using species-specific primers (2.5 pmol), 1.5 mM $MgCl_2$ and at annealing temperature at 55°C. The outcome of the experiment concluded that only indicator pathogens *P. aeruginosa*(709 bp), *E. coli* (559 bp), *S. aureus*(461 bp), and *Salmonella* spp., (275 bp) were amplified and absence of non-specific bands were pictured as shown in Fig 2. We also amplified with the mixture of other pharma contaminants DNA and no bands were seen in Fig 3.

We attempted to test the different strains of *E.coli*(4), *S. aureus*(3), *P. aeruginosa*(3), *Salmonella* spp. (4) and other non-indicator pathogens (21) for determining the specificity of our primers. The results of the strain-specific PCR were tabulated in Table (2).

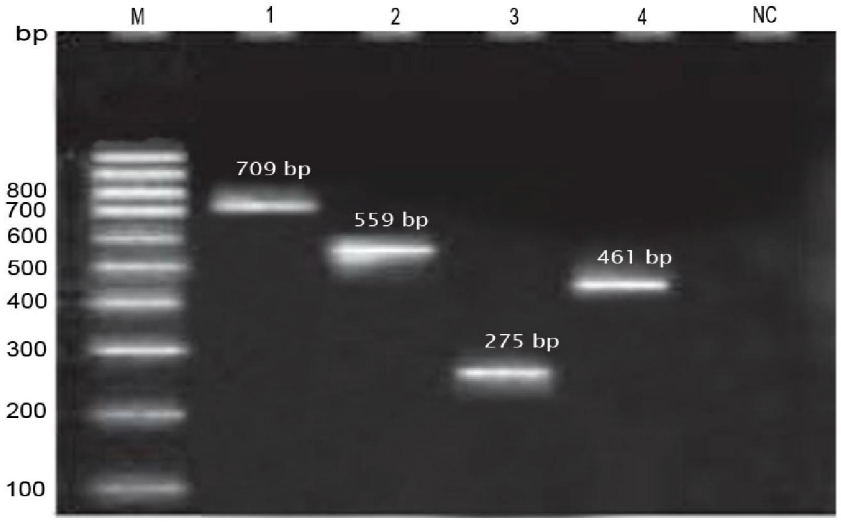

Fig. 1: Uniplex PCR assay. Lane 1, *P. aeruginosa* (709 bp) product when amplified with DNA of *P. aeruginosa*; lane 2, *E. coli* (559 bp) product when amplified with DNA of *E.coli*; lane 3, *S.*spp. (275 bp) product when amplified with DNA of *S. typhimurium*; lane 4, *S. aureus* (461 bp) product when amplified with DNA of *S. aureus*; NC, negative control; M, marker (100 bp ladder)

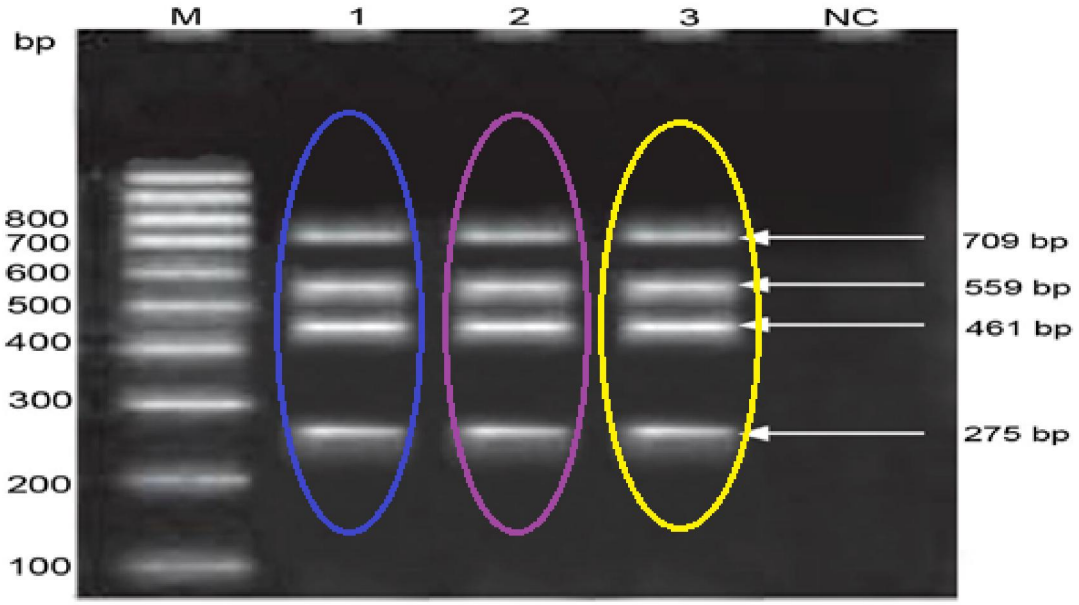

Fig. 2: Multiplex PCR assay, Lane 1,2 and 3, *P. aeruginosa*(709 bp); *E. coli* (559 bp); *S. aureus*(461 bp); *S. typhimurium* (275 bp); NC, negative control; M,marker (100 bp ladder).
Specificity of multiplex PCR

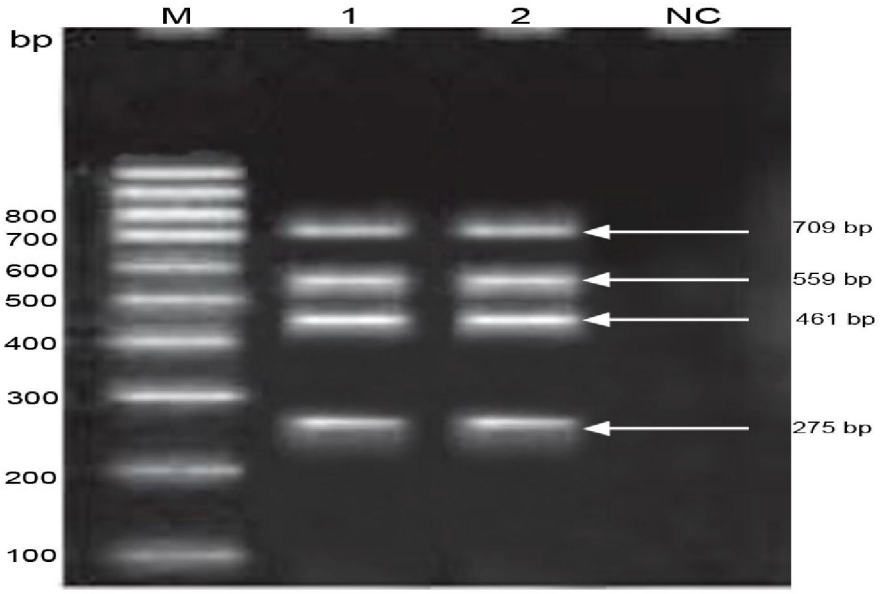

Fig. 3: Specificity of multiplex PCR; lane 1 and 2*, P. aeruginosa*(709 bp); *E. coli* (559 bp); *S. aureus*(461 bp); *S. typhimurium* (275 bp); NC, negative control; M, marker (100 bp ladder)

Sensitivity of multiplex PCR by DNA concentration

The sensitivity of multiplex PCR method was regulated by varying DNA quantity (25–0.39 ng) of each pathogen *P. aeruginosa*, *E. coli*, *S. aureus*, *S. typhimurium* , 2.5 pmol primers, 1.5 mM $MgCl_2$and annealing temperature at 55°C. All the indicator pathogens were detected even at 1.56 ng of DNA quantity. There was gradual or distinct decrease in the band intensity when used DNA from higher concentration (25 ng) to lower concentration (1.56 ng) as shown in Fig. 4.

Table (2): Specificity of the primers with different bacterial strains.

Bacterial strains	PCR results – tested with specific primers			
	Escherichia coli	*Staphylococcus aureus*	*Salmonella*	*Pseudomonas aeruginosa*
E. coli **([a]ATCC 11775)**	+	-	-	-
E. coli **(ATCC 8739)**	+			
E. coli **(ATCC 25922)**	+			
E. coli **(ATCC 8739)**	+			
S. aureus **(ATCC 25923)**		+		
S. aureus **(ATCC 6538)**		+		
S. aureus **(ATCC 12600)**		+		
S. typhimurium **(ATCC 6994)**			+	
S. typhimurium **(ATCC 14028)**			+	
S. typhi **(ATCC 19430)**			+	
S. paratyphi **(ATCC 9150)**			+	
Ps. aeruginosa **(ATCC 27853)**				+
Ps. aeruginosa **(ATCC 9027)**				+
Ps. aeruginosa **(ATCC 10145)**				+
P. mirabilis **(ATCC 29906)**				
P. vulgaris **(ATCC 13315)**				
M. morganii**(25830)**				
P. rettgeri**(ATCC 29944)**				
B. cereus **(ATCC 14579)**				
C. freundii**(ATCC 8090)**				
E. aerogenes (**ATCC 13048)**				
E. agglomerans**(ATCC 27155)**				
E. sakazaki**(ATCC 29544)**				
H. influnzae**(ATCC 33391)**				
H. pylori **(ATCC 43504)**				
K. pneumonia **(ATCC 13883)**				
S. marcescens**(ATCC 13880)**				
S. pyogenes**(ATCC 12344)**				
S. flexneri**(ATCC 29903)**				
S. sonnei**(ATCC 29930)**				
Y. enterocolitica (**ATCC 9610)**				
B. substilis**(ATCC 6633)**				
E. cloacae **(ATCC 23355)**				
E. faecalis**(ATCC 29212)**				
S. epidermidis**(ATCC 12228)**				

[a] ATCC: American Type Collection Culture, Rockville, MD, USA.

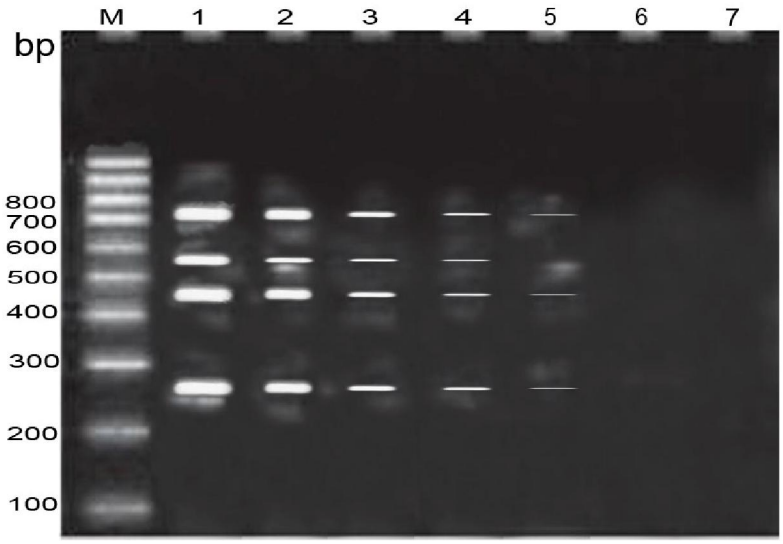

Fig. 4: Sensitivity of multiplex PCR was indicated by varying indicator pathogen DNA concentration. Lanes1–7: 25, 12.5, 6.25, 3.12, 1.56, 0.78 and 0.39 ng respectively; M, marker (100 bp ladder).

Sensitivity of multiplex PCR by bacterial cultures using multiplex PCR.

Indicator pathogen cultures were subjected to multiplex PCR with 2.5 pmol primers, 1.5 mM $MgCl_2$ concentrations and annealing temperature at 55°C. Our analysis revealed that multiplex PCR were able to determine 10 CFU/g of all the four indicator pathogens (*Ps. aeruginosa, E. coli, S. aureus* and *Salmonella* spp.) with distinct amplicon sizes (709, 559, and 461, 275 bp respectively) in artificially contaminated pharmaceutical products. The results were shown in figure (5). The reproducibility of results with the use of multiplex PCR was done two times to demonstrate the reproducibility of PCR products.

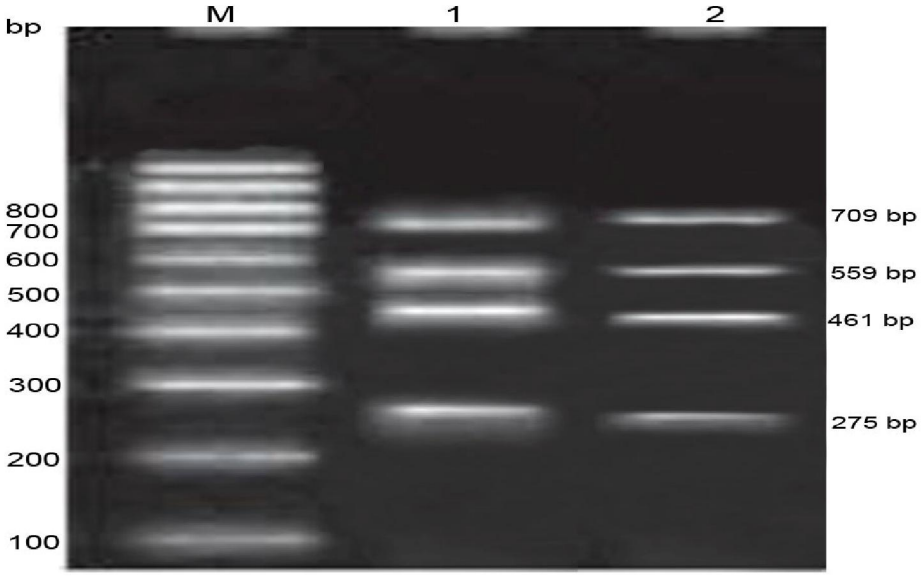

Fig. 5: Sensetivity of multiplex PCR by bacterial cultures. Lane 1, *P. aeruginosa*(709 bp); *E. coli* (559 bp); *S. aureus*(461 bp); *S. typhimurium* (275 bp) products, when amplified with (20 CFU/ml) of bacterial cultures; lane 2, *P. aeruginosa*(709 bp); *E. coli* (559 bp); *S. aureus*(461 bp); *S. typhimurium* (275 bp) products, when amplified with (10 CFU/ml).of bacterial culture; M, marker (100 bp ladder).

Detection of indicator pathogens in finished pharmaceutical products using multiplex PCR assay

Fifty pharmaceutical samples were tested using mPCR ; where twelve syrup samples, sixteen tablet samples and twelve capsule samples in addition to four samples for topical and six stored samples. Our results revealed that all pharmaceutical samples gave positive PCR results which mean that no masking or interfering effect exerted from the products samples.

Hundred percentage correlations between standard conventional USP method of analysis & the PCR method was obtained, in which standard methods gave the same as PCR results despite the rapidity of the latter technique.

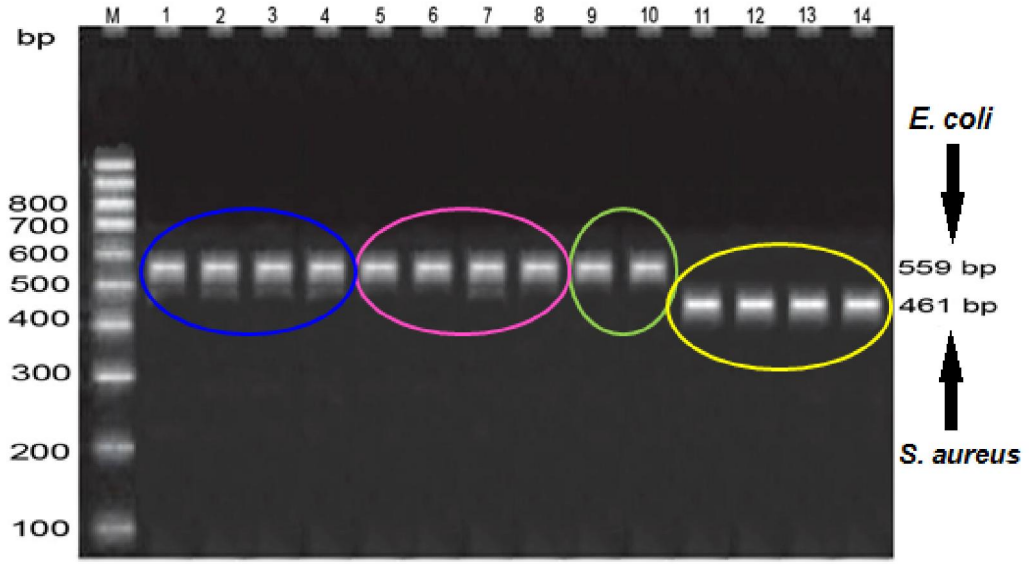

Fig. 12: Representative multiplex PCR assays for detection of indicator pathogens in finished pharmaceutical product. Lanes: M, marker (100 bp ladder). Lanes 1-4, four syrup samples; lanes 5-8, four tablet samples; lanes 9-10, two capsule samples and lanes 11-14; four topical samples.

4. Discussion

Strict regulations govern the production of pharmaceutical products whether they are sterile or nonsterile. Certain official tests are carried out in microbiology testing laboratory in any pharmaceutical production facility to ensure the pharmaceuticals microbiological quality according to the standard pharmacopeial recommendations. Non sterile products must be free of specified microorganisms that are used as a check for their quality. Oral preparations must be free of *Salmonella spp* and *E. coli* and topical preparations must be free of *P. aeruginosa* and *S. aureus,* and Conventional microbiological methods are time-consuming, labor-intensive, and require long incubation times, resulting in delaying the release of the products. Notable progress has been made in methods that encourage the use of PCR as a rapid and accurate tool in microbiological testing of pharmaceuticals (Raghe*bet al*., 2012). Recently, molecular assays such as nucleic acid hybridization techniques (DNA probes) and PCR have been developed. PCR has been shown to combine rapid results and high specificity in detecting both pathogenic and spoilage microorganisms (Lampel*et al*., 2000 and Raghe*bet al*.,2012). This represented a faster turn over time than the standard 5–6 days detection time.

In this study PCR analysis was used for detecting the presence of USP bacterial indicators in pharmaceutical products samples. Oral samples were tested for the presence of *S. aureus, E.coil, Ps. aeruginosa*&*Salmonella. Spp.*. Results showed that all products had no interfering effect on the PCR assay. Furthermore, all tested samples gave negative PCR results i.e. samples free of the indicator bacterial strains which were consistent with standard USP results. These results in agreement with Jimenez *et al.*(2001) work that use the BPW as preenrichment medium instead of LB. Using of LB as preenrichment medium will not allow *S. typhimurium*detection in the presence of the other bacterial species which can outcompete *S .typhimurium.* Preenrichment in low nutrient BPW medium increase *S. typhimurium*densities to the levels required for PCR detection.

In our study, we could able to optimize the detection time for *E. coli*, *Salmonella* spp., *S. aureus*, and *P. aeruginosa*from 5– 7 days to less than 24 h,

and simultaneous detection of all four indicator pathogens by multiplex PCR was performed on all artificially contaminated samples. During manufacturing, microorganisms are subjected to processes that might kill or reduce microbial densities. Furthermore, finished products contain preservatives and other antimicrobial components which injure microbes sub-lethally and in some cases require a low nutrient enrichment media to enhance microbial growth. Sample pre-enrichment is the most vital step during isolation of *Salmonella* spp. from pharmaceutical samples. To optimize *S. typhimurium*PCR detection, particularly in minimal bacterial load samples, a different type of pre-enrichment broth was performed. BPW was previously used to enhance the recovery of *Salmonella* spp. in food samples using conventional and PCR methods. (Gouws*et al*., 1998).

Ray, (1986) reported that the importance of resurgence and recovery procedures availed in food industry has been overlooked in the isolation techniques of pharmaceutical microbiological studies. When pharmaceutical raw materials and products contaminated with mixed bacterial cultures of *E. coli*, *P. aeruginosa*, *S. aureus*, and *S. typhimurium*were pre-enriched in lactose broth with and without tween-20, 10 (40%) of 25 samples did not show the presence of the *Salmonella* spp. The presence of non-*Salmonella* bacteria influenced the performance of the PCR-based assay was reported Jimenez *et al*., 2001. But when the same samples were enriched in BPW, all the samples were shown positive for *Salmonella*. Ray, 1986 revealed that all ten samples that were shown PCR negative in lactose broth were found to be positive with BPW enrichment medium. BPW enrichment medium increased *S. typhimurium*bacterial growth resulting in rapid PCR detection. In this study, we used trypticase soy broth containing 4% Tween-20 and 0.5% soy lecithin for enrichment of *E. coli*, *S. aureus*, and *Ps. aeruginosa*and BPW for *S. typhimurium*.

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. After the enrichment step, sufficient bacteria were grown and allowed the pathogens to be detected by PCR when the original sample had ·10 CFU/ ml. In current study, DNA was extracted from contaminated sample suspensions by using the phenol- chloroform- isoamyl alcohol method. With the latest advances in microbial genomics, the availability of primer sequences are limitless, allowing in selecting targets from different loci of bacterial genome reported (Jimenez *et al.,* 2001). In this study, we have designed primers. The primer sequence, the target, and the PCR product sizes were summarized in Table 1. Initially the uniplex PCR conditions were standardized and revealed that the primers are very sensitive to detect the specific organisms. We further developed multiplex PCR where all four PCR primers have similar melting temperatures (Tm 55 °C) which will simultaneously detect all indicator pathogens. Our current studies were in agreement with the findings of Henegariu*et al.* (1997) that the relative concentrations of the primers were found to be the most important factor in determining approximately equal yields of amplification products from of the each organism in a single reaction.

Uniplex PCR was used in this study, which further elevated to multiplex PCR for simultaneous detection of indicator pathogens that were evaluated microbiological test for their ability to accurately identify the recovered pharmaceutical products containing antimicrobial preservatives. The results revealed that the primers were very sensitive and specific to detect bacterial pathogens by adopting annealing temperature at 55 °C, 10 ng of DNA, 5 pmol of primer and 1.5 mM $MgCl_2$ concentration Since, uniplex PCR depicted promising results, and for the reason that all pathogens were detected at similar annealing temperature (55° C), we devised multiplex PCR.. The uniplex PCR amplicons were successfully sequenced, confirming the conservation of used primers. Other validation parameters such as specificity, sensitivity, and robustness were examined closely.These results were similar toRagheb*et al* (2012), they reported that Uniplex PCR was performed for the detection of each microorganism individually targeting the conserved region in each bacterial genome.

A major effort, during this study was directed to determine the feasibility of extending the convenience, accuracy, and reproducibility of multiplex PCR for identification of contaminated pharmaceutical finished products. The multiplex PCR results determined the presence of four bacterial pathogens in single reaction with varying amplicon sizes *P. aeruginosa*(709 bp)*; E. coli* (559 bp)*; S. aureus*(461 bp); *Salmonella* spp. (275 bp), respectively. Therefore, The current study demonstrates the sensitive methodology to detect indicator pathogens with minimum quantity of DNA. The multiplex PCR was a useful and reliable tool especially for laboratories lacking the equipment or personnel with expertise to apply conventional methods to identify indicator pathogens from non-sterile pharmaceutical products. Similarly, Jimenez *et al.* (2001) studied the detection time of *E. coli*, *S. aureus, P. aeruginosa*, and *A. niger*in cosmetic/

pharmaceutical samples and reduced from 6–8 days (standard methods) and by PCR analysis to 24–27 h.

Jimenez, 2001 in previous studies for the detection of *E. coli*, *S. aureus*, *P. aeruginosa*, and *A. niger*in pharmaceutical samples, simultaneous detection of the pathogens (multiplex PCR) were attempted using RoboCycler 96-gradient PCR with different annealing temperatures of primer profile for *E. coli* (54 °C), *S. aureus*(65 °C), *P. aeruginosa*(55 °C), and *A. niger*(62 °C), respectively, was reported. In that study they attempted in using different annealing temperatures for detecting *E.coli*, *S. aureus*, *P.aeruginosa*, and *A. niger*, and moreover gradient PCR in a single PCR run was reported. In current study, we formulated the all four primers will anneal at single temperature (55 °C) not deviating more than ±5 °C. By simultaneously amplifying more than one locus in the same reaction, multiplex PCR was becoming a rapid and convenient screening assay in both the clinical and the research laboratory (Henegariu, *et al*., 1997). Little is known about the factors and common difficulties influencing a multiplex PCR. Other critical factors in multiplex PCR include the concentration of the PCR buffer, the balance between the magnesium chloride and deoxyribonucleotide triphosphate concentrations and the cycling temperatures (Henegariu, *et al*., 1997).

Our analysis revealed that multiplex PCR were able to determine 10 CFU/g of all the four indicator pathogens (*Ps. aeruginosa, E. coli, S. aureus* and *Salmonella* spp.) with distinct amplicon sizes (709, 559, and 461, 275 bp respectively) in artificially contaminated pharmaceutical products. These results in agreement with Jimenez (2001) showed simultaneous detection of *E.coli*, *S. aureus*, *P.aeruginosa*, and *A. niger*with detection levels <10 CFU/g or ml using RoboCycler 96-gradient PCR was reported. But in the present study twin goals were achieved; first the detection limits of multiplex PCR were found to be 10 CFU/g or ml, secondly multiplex PCR method improved the sensitivity of the detection limit by utilizing minimum quantity of DNA (1.56 ng) for all the four indicator pathogens.

In the present study to detection of indicator pathogens in finished pharmaceutical products using multiplex PCR assay**;** fifty pharmaceutical samples were tested using mPCR ; where twelve syrup samples, sixteen tablet samples and twelve capsule samples in addition to four samples for topical and six stored samples. Our results revealed that all pharmaceutical samples gave positive PCR results which mean that no masking or interfering effect exerted from the products samples. Hundred percentage correlations between standard conventional USP method of analysis & the PCR method was obtained, in which standard methods gave the same as PCR results despite the rapidity of the latter technique.

Multiplex PCR method is rapid and the level of sensitivity achieved in our experiments is applicable to the practical survey of microbial contamination in pharmaceutical samples. A major outcome of the study is the use of a multiplex PCR to detect multiple pathogens using compatible primers and the DNA extracts from the pharmaceutical samples. The implications of the present study are promising and choice of primers in PCR can be extended to detect indicator pathogens present in pharmaceuticals and cosmetic finished products. The use of molecular analyses such as PCR and multiplex assays have resulted in optimization of product manufacturing, quality control evaluation, and product release in cosmetic and pharmaceutical laboratories in few instances. When compared with standard methods, these technologies provide rapid and reliable microbiological monitoring of finished products. Molecular methods have enhanced the ability of an industrial microbiology laboratory to rapidly assess system breakdowns and quality processes.

To optimize pharmaceutical process control, corrective actions must be performed in real time, not after 7 or more days of manufacturing. Rapid methods will identify microbial contamination with detection times ranging from 90 min to 30 h allowing the monitoring of critical control points, reducing losses, and optimizing resources. A recent technical report by the Parenteral Drug Association (PDA) provided some information and guidelines for the evaluation, validation, and implementation of rapid microbiological methods (PDA, 2000). Further developments in rapid method technologies might lead to online monitoring of pharmaceutical manufacturing and environments.

Samples of topical products were analyzed using PCR for the *Ps.aeruginosa,* and *S. aureus*detection. Ten grams or ml of samples were added into TSB & another 10 g or ml of each product were artificially contaminated with standard control strains to exclude the inhibitory effect of the products samples on bacteria. After 24 hrs incubation period, DNA extraction, PCR amplification and agarose gel electrophoresis were performed. All products had no interfering effect on the PCR assay, furthermore; all tested samples gave negative PCR results i.e. samples free of the indicator bacterial strains. This work was consistent with Jimenez *et al* (1999) one, except for using 10% product enriched broth in our work instead of 2% product enriched broth with the same level of the spiked bacteria i.e. 10-100cfu/g or ml of sample. While Jimenez *et al.* (2000) develop another PCR assay to detect lower contamination level by inoculating less than 10 cfu/ml or g of products with

*E. coli, Ps. aeruginosa, S. aureus*and *A. niger* separately into preenrichment broths with 10% product. Therefore, higher volumes of the extracted lysate were analyzed for PCR detection. Jimenez *et al.* (1999 & 2000) used PCR beads for *E. coli, Ps. aeruginosa, S. aureus*detection which contains the necessary PCR reagents without DNA primers, & Jimenez *et al.* (2001) used a tablet form contains PCR reagents with *S. typhimurium*DNA primers. These PCR beads & tablets reduce the time consuming handling & preparation of PCR reagents. In the present study, conventional PCR reagents were prepared due to its availability & it gave the same results. Merker*et al*. (2000), employed Fluorescence-coupled PCR technology to quantify DNA segments specific for S. *aureus, P aeruginosa, and Enterobacteriaceae.* His results were consistent with the present work i.e. 100% correlation between PCR assay & standard procedures was found.

5. Conclusion

Our study was concluded that;

1- Multiplex PCR assay provides sensitive and reliable results and allows for the cost-effective detection of all four bacterial pathogens in single reaction tube.

2- In conclusion, based on our findings, the mPCR assay provides a specific method for simultaneous evaluation and detection of low levels of USP bacterial indicators in our test sample and helps the optimization of product manufacturing, quality control and release of pharmaceutical products in a timely manner.

3- The application of mPCR technology in microbial quality control of non sterile pharmaceutical products can be performed in rapid & accurate detection of objectionable microorganisms and allows for the cost-effective detection of all bacterial pathogens and timely manner in pharmaceutical industry, which leads to faster release of products and more rapid implementation of corrective actions. However; PCR detection of bacteria required 27 hr while standard methods completed within 6-8 days. This rapid quality evaluation resulted in optimization of product manufacturing, quality control & release of finished products.

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