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DETECTION OF *BREVUNDIMONAS DIMINUTA* BY POLYMERASE CHAIN REACTION IN MEMBRANE FILTER VALIDATION

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ABSTRACT

The aim of present work was to develop the PCR based microbial testing method for detection of *Brevundimonas diminuta* (ATCC 19146) for Bacterial challenge test and compare the results of the conventional microbial testing method and the PCR based microbial testing method. It involved the preparation of the sample for the conventional and PCR based microbial testing, microbial testing by conventional method, PCR based microbial testing (extraction of DNA from bacterial cell and PCR thermal cycling, visualization of PCR product by agarose gel electrophoresis and comparison of results of conventional microbial testing method with PCR based microbial testing method. This technical report is intended to provide a systematic approach to selecting and validating the most appropriate filter for a sterilizing filtration application. Early, careful screening of potential filter types and configurations can result in fewer technical and regulatory problems, fewer delays, more efficient product processing and greater sterility assurance.

KEYWORDS: PCR, *Brevundimonas diminuta*, PCR thermal cycling, agarose gel electrophoresis, sterilizing filtration.

INTRODUCTION

Sterilization is complete eradication of all forms of life along with microbial spores. Various methods of sterilization are used in the pharmaceutical industry. Sterilization filtration is one of the several methods that are widely used. Sterilizing filtration is the process of removing all microorganisms, excluding viruses, from a fluid stream. A sterilizing grade filter must

remove all microorganisms present in a fluid stream without adversely affecting product quality.

In the early 1900s, the first parenteral drugs were manufactured on an industrial scale. The need arose to find a suitable sterilization method for heat-sensitive products that could not be autoclaved in the final container, i.e., had to be aseptically processed. Later, filtration to remove sub visible particulates from parenteral preparations, particularly solutions introduced intravenously, was found to be important.[1]

Initially, porcelain filters by Chamberlain were used in pharmaceutical production. However, problems with cleaning these permanent filters and the danger of cross-contamination led to their replacement. The first filter medium to be used on an industrial scale worldwide for almost 50 years was a cellulose-asbestos filter known as the Seitz EK Filter (EK - Entkeimung, "germ removal") .Since the mid-1970s this filter has been forced off the market because of the asbestos fiber issue. The forerunner to the membrane filter was developed by Zsigmondy and Birchmann who patented a graded series of membranes in 1918. The Sartorius-Werke Aktiengesellschaft, Gottingen, Germany, refined the Zsigmondy process and in 1929 began the commercial production of membrane filters on a small scale. The first membrane disks led to the replacement of porcelain filter cartridges in pharmaceutical production. There still was a lack of efficient, large surface area, inexpensive membrane filters for use in large parenteral batch production. The membrane filter cartridges, especially the pleated cartridges that entered the market in the 1970s, were a step in the right direction. There still were problems, as the retention rate for bacteria was unacceptable to the pharmaceutical industry. The problem was examined systematically, and a collaboration between filter manufacturers and pharmaceutical companies developed basic principles that would ensure safety in sterilization by filtration. The homogeneity of the membranes in the sponge-like labyrinth system and the concern about the potential for interfering "large pores" initially led to the use of double-layer membranes. Additionally, defects in the cartridges arose from the folding and welding, or gluing of the membranes, as well as defective seals and damage during autoclaving. Newly developed integrity test equipment helped, allowing accurate measurements to assess these defects. Improved filter manufacturing techniques eventually eliminated these defects.[2]

Until the late 1960s, 0.45 m-rated membranes were considered "sterilizing grade" filters, and were used successfully in the sterilizing filtration of parenteral. Such filters were qualified using 0.6x1 m *Serratia maracescens*, a standard bacterium for qualifying analytical membranes used for water quality testing (ASTM, 1980). In the mid-1960s, however, Dr. Frances Bowman of the FDA observed a 0.45 m "sterile-filtered" culture medium to be contaminated with an organism, subsequently shown to penetrate 0.45 m-rated membranes repeatedly in small numbers at challenge levels above 104-106 per cm² (Bowman et al., 1967). Bowman also observed that the next finer grade commercial membrane (nominally 0.22 m-rated) effectively retained this organism at similar challenge levels. This 0.3x0.6-0.8 m contaminant was identified as *Pseudomonas diminuta* (currently reclassified as *Brevundimonas diminuta*), and registered with the American Type Culture Collection (ATCC) as Culture No. 19146. This strain has been accepted widely by filter manufacturers and industry as the standard challenge organism for qualifying sterilizing grade membrane filters (ASTM, 1983; ISO, 1995).[3-4]

Following the broad acceptance of *B. diminuta*, FDA incorporated demonstration of its retention in the definition of a sterilizing filter. Selecting a sterilizing grade filter requires consideration of many important issues, such as materials of construction and their compatibility with the product. The selection also should consider the processing characteristics, including the volume of product filtered, flow rate, pressure differential, temperature and the chemical characteristics of the product.

Current available materials of construction include, but are not limited to polymers such as cellulose esters, nylon, polyesters, polytetrafluoroethylene, polyvinylidene fluoride, polycarbonate, polypropylene, polyethersulfone and polysulfone. Generally, Hydrophilic filters are used for aqueous based liquid processes, while hydrophobic filters are used for solvent, vent and gas applications. Factors influencing the correct choice of filter include chemical compatibility, thermal stress resistance, hydraulic stress resistance, toxicity testing, bacterial challenge, physical integrity testing. The need to control microbial bioburden has led to point-of-use bacterial sterilization using filter membranes to remove particles and bacteria from critical fluids. The microbial rating of a membrane filter assembly is determined by its ability to retain particle sizes represented by specified strains of microorganisms. Bacterial challenges are typically performed during validation to evaluate membrane filter performance. Verification of bacterial removal is typically performed by

challenging a membrane filter assembly with a suspension of a known organism. The titer reduction is the ratio of influent colony forming units (CFU) to effluent bacteria colonies found downstream of the membrane.[5-8]

During filter evaluation, it is best to consider all processing variables so the appropriate filter surface area and configuration can be selected. There are three basic types of filter configurations generally accepted for sterilizing products: flat stock membranes, preassembled capsules and membrane cartridge assemblies.

TYPES OF FILTRATION

Membrane filtration has become a key element for use in the sterilization of fluids (both gases and liquids) during aseptic processing of pharmaceutical solutions.

Particle removal by membrane filtration may be simply classified as follows:

Table 1

Classification of filtration on basis of size.

TYPE	RELATIVE SIZE MICRONS	SUBSTANCE REMOVED
Macro filtration	10 to 1000	Visible Particles
Microfiltration	0.1 to 10	Bacteria
Ultrafiltration	0.001 to 0.1	Viruses,Pyrogens,Proteins
Nanofiltration	0.0005 to 0.001	Aqueous Salts, Small molecules
Reverse osmosis	0.0001 to 0.00005	Metallic ions

Directionally, there are two kinds of membrane filtration.

1) Vertical or direct flow filtration: Microfiltration of pharmaceutical solutions is primarily direct flow filtration. It is also called as a depth filtration.
2) Horizontal or cross flow filtration: Cross flow filtration primarily an Ultrafiltration tech. used in protein and biological material separations.

   The various types of membranes that can be used are spiral, ceramic, stainless steel, tubular, hollow fibre, plate and frame.[9-12]

MATERIALS AND METHODS

Taq Polymerase enzyme and buffer E, DNTP mix, DNA ladder, DNA GeNei^{TM} Spin Genomic DNA Prep Kit, Ethidium bromide and gel loading buffer were made availble from Bangalore Genei. Forward Primer and Reverse Primer were received from Ocimum Biosolutions (India) Ltd. Hyderabad. Agarose for electrophoresis was obtained from Sisco

Research Laboratories Pvt. Ltd. The strain of *Brevundimonas diminuta* (MTCC No.1287) was procured from MTCC chandigarh.

(i) Method of isolation of DNA for positive control

For the extraction of the DNA, GeNei^{TM} Spin Genomic DNA Prep Kit (From Bacteria) from the Bangalore Genei was used. A nutrient broth strain of Brevundimonas diminuta was prepared. 5 ml broth strain of *Brevundimonas diminuta* was centrifuged at 5000 rpm to form pellet. To these pellet 1 ml of PBS was added and then spined followed by draining, repeated three times. Bacterial pellet of appropriate culture volume was resuspended in 180 µl of lysis buffer I. Proteinase K 20 µl was added and mixed thoroughly by vortexing and then incubated at 55oC for 1 hour. The sample was vortexed at intervals for better lysis. The sample was spined at maximum speed in a microcentrifuge for 5 minutes and the supernatant decanted carefully to a fresh vial. To the supernatant 200 µl of Lysis buffer II was added and mixed thoroughly by vortexing and incubating at 70oC for 20 minutes. 4 µl of RNase (100 mg/ml) was added, mixed by vortexing and incubated at room temperature for 5 minutes. The sample was spined at maximum speed in a microcentrifuge for 5 minutes and the supernatant decanted carefully to a fresh vial. To the supernatant 200 µl of distilled ethanol was added and mixed thoroughly by vortexing. The spin column was kept in a 2 ml collection tube and the sample-ethanol mixture was added. It was then centrifuge at maximum speed for 1 minute and the collection tube with flow through was discarded. The spin column was kept in a fresh 2 ml collection tube and 500 µl of wash buffer I was added and spined at maximum speed for 1 minute and collection tube with wash sample was discarded. The spin column was kept in a fresh 2 ml collection tube and 500 µl of wash buffer II was added and spined at maximum speed for 3 minute and wash fraction discarded and collection tube retained. The empty column was spin for two minutes at maximum speed to ensure the removal of wash buffer. The spin column was placed in a fresh 1.5 ml tube and 200 µl of pre warmed elution buffer was added and incubated at room temperature for 5 minutes and centrifuged for 2 minutes to elute the DNA.

(ii) Isolated DNA confirmation by Agarose Gel Electrophoresis.

1 % Agarose with ethidium bromide in 0.5 X TAE was prepared and transferred to submerged Agarose gel electrophoresis apparatus and allow to cool to form a gel .The comb was removed and then the electrophoresis buffer (0.5 X TAE) was added. Then 10 µl isolated DNA with gel loading buffer was loaded in the well. The gel was run at a constant voltage

(100 volt.) for 45 minutes and monitored by the tracer present in the gel loading buffer. The isolated DNA bands were observed in Gel documentation instrument.

(iii) Quantitation of DNA by UV Spectrophotometric method

1 µl from each isolated DNA of five different strains was dissolved in 500 µl double distilled water. The OD values on spectrophotometer were taken at 260 nm. The amount of DNA were quantified using the formula DNA concentration (ng/ml.) = OD_{260} x 500 (dilution factor) x 50 µg/ml.[13-19]

(iv)PCR Thermal Cycling for positive control

The PCR thermal cycling of strain of *Brevundimonas diminuta* which was previously subjected to the DNA extraction procedure and the negative control was done as follows.

Table 2: A typical 25 µl PCR reaction.

Reagent	Quantity		
	Sample A	Sample B	Control
Volume	5 µl	5 µl	-
dNTPs	2 µl	2 µl	2 µl
Taq buffer	2.5 µl	2.5 µl	2.5 µl
Taq polymerase	0.3 µl	0.3 µl	0.3 µl
Forward Primer	0.5 µl	0.5 µl	0.5 µl
Reverse Primer	0.5 µl	0.5 µl	0.5 µl
Distilled water	14.2 µl	14.2 µl	19.2 µl

The above mixture was subjected to the PCR thermal cycling. The above mixture was first of all subjected to 94^{o}C for 5 minutes and then the 30 cycles at different temperatures as shown in the table 6 were run by setting the programmed on the PCR thermal cycler.[20-24]

Table 3: PCR Conditions

Process	Temperature ^{o}C	Time
Initial denaturation	94 ^{o}C	5 minutes
Denaturation	94 ^{o}C	30 second
Annealing	50 ^{o}C	30 second
Extension	72 ^{o}C	30 second
Incubation	72 ^{o}C	7 minutes
Cooling	4^{o}C	5 minutes

(v) For microbial testing in ophthalmic solutions.

0.1 ml nutrient broth solution A of *Brevundimonas diminuta* (ATCC NO. 19146.) was transferred in the conical flask containing 100 ml of saline solution (B). 0.1 ml of solution was transferred from B to the other conical flask containing 100 ml of saline solution (C -10^3 dilutions). Then 0.1 ml of solution was transferred from C to the other conical flask containing 100 ml of saline solution (D -10^6 dilutions). Again 0.1 ml of solution was transferred from D to the other conical flask containing 100 ml of saline solution (E -10^9 dilutions). Again 0.1 ml of solution was transferred from E to the other conical flask containing 100 ml of saline solution (F -10^{12} dilution). In this way the dilutions of *Brevundimonas diminuta* (ATCC NO. 19146.) in the saline solution were made. The 100 ml solution A was passed on 0.2 um filter at 760 mM Hg subjected to filtration.[25-30]

The membrane filter was washed with PBS solution for the collection of micro-organism (Sample A). Filtrate was again filter and the membrane filter was washed with PBS solution for the collection of micro-organism in the filtrate. (Sample B). Both the samples were subjected to PCR.

The PCR thermal cycling of Sample A and Sample B prepared as above and the negative control was done as follows.

Table 4: A typical 25 µl PCR reaction

Reagent	Quantity		
	Sample A	Sample B	Control
Volume	5 µl	5 µl	-
dNTPs	2 µl	2 µl	2 µl
Taq buffer	2.5 µl	2.5 µl	2.5 µl
Taq polymerase	0.3 µl	0.3 µl	0.3 µl
Forward Primer	0.5 µl	0.5 µl	0.5 µl
Reverse Primer	0.5 µl	0.5 µl	0.5 µl
Distilled water	14.2 µl	14.2 µl	19.2 µl

The above mixture was subjected to the PCR thermal cycling. The above mixture was first of all subjected to 94^{o}C for 10 minutes and then the 30 cycles at different temperatures as shown in the table 8 were run by setting the programmed on the PCR thermal cycler.

Table 5: PCR conditions

Process	Temperature ^{o}C	Time
Initial denaturation	94 ^{o}C	10 minutes
Denaturation	94 ^{o}C	30 second
Annealing	50 ^{o}C	30 second
Extension	72 ^{o}C	30 second
Incubation	72 ^{o}C	7 minutes
Cooling	4 ^{o}C	5 minutes

(v) Visualization of PCR product by Agarose gel electrophoresis

The method adopted for the visualization of PCR product of both i.e. positive control and microbial testing in ophthalmic solutions remains the same, which is carried out as described- 1 % Agarose with ethidium bromide in 0.5 X TAE was prepared and transferred to submerged Agarose gel electrophoresis apparatus and allow to cool to form a gel .The comb was removed and then the electrophoresis buffer (0.5 X TAE) was added. The standard 100 bp ladder was loaded in first well and then all 25 µl of PCR product in each PCR tubes with gel loading buffer (2 µl) were loaded in subsequent wells. The gel was run at a constant voltage (100 volt.) for 45 minutes and monitored by the tracer present in the gel loading buffer. The isolated DNA bands were observed in Gel documentation instrument.

RESULTS AND DISCUSSION

Conventional Microbial Testing: The CFU of Sample A was observed 4 x 10^{12}. No colonies were observed in sample B. PCR Thermal Cycling for positive control: The Oligonucleotide sequences which are used in the PCR thermal cycling for the amplification of DNA , the strain of *Brevundimonas diminuta*, hence PCR based microbial testing developed by us can detect strain of *Brevundimonas diminuta* in the sample.

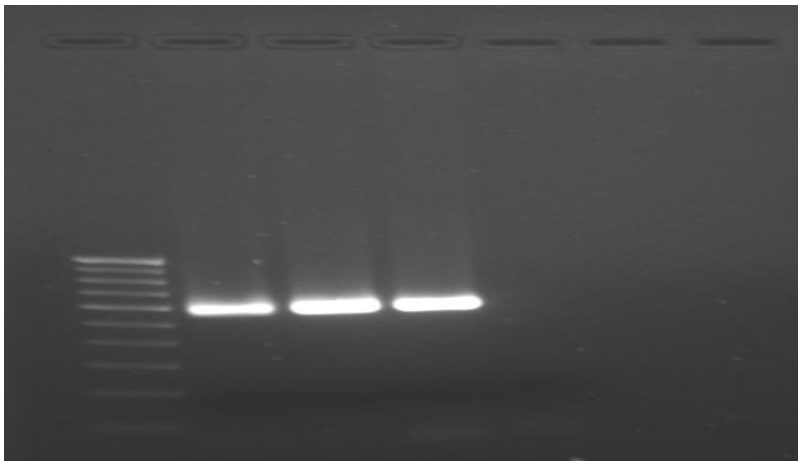

Figure 1 PCR Thermal Cycling result for DNA isolated from strain of *Brevundimonas diminuta.*

Lane 1- DNA ladder

Lane 2- 1 µl DNA (ATCC.19146)

Lane 3 -2 µl DNA (ATCC.19146)

Lane 4 -4 µl DNA (ATCC.19146)

Lane 5 –Control.

PCR Thermal Cycling for microbial testing of Sample A Sample B: After PCR thermal cycling **Sample A** and **Sample B** run on agarose gel electrophoresis.

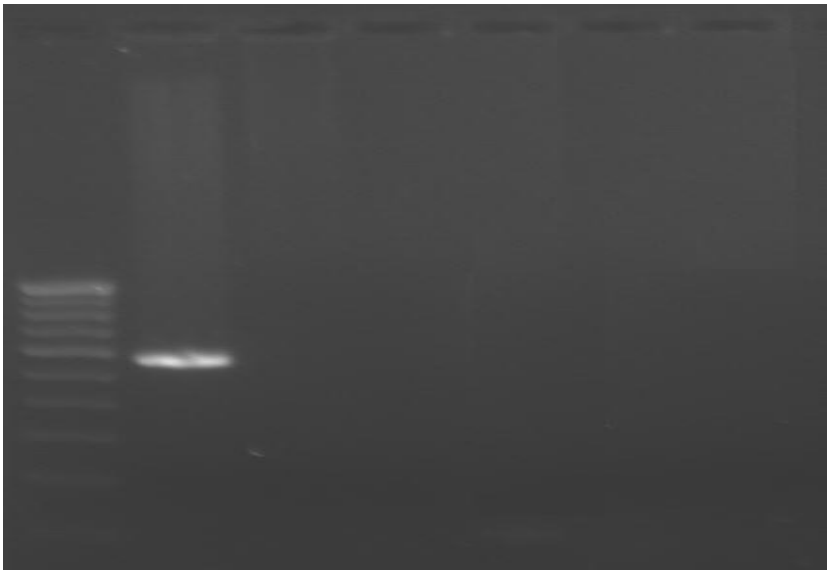

Figure 2 PCR thermal cycling result of Sample A and Sample B after filtration.

Lane 1- DNA ladder

Lane 2- Sample A

Lane 3 – Sample B

Lane 4 -Control

1. Figure 1 showing band of isolated DNA of *Brevundimonas diminuta* (ATCC No.19146). The third lane on the agarose gel was of isolated DNA band. These resµlts confirmed the DNA of *Brevundimonas diminuta* isolated from source.
2. The figure 2 Showing different bands of different concentrations of *Brevundimonas diminuta* positive control. The first lane on the agarose gel was of 100 bp standard DNA ladder, hence it showed different bands at 100 bp intervals. The second third and fourth lanes were of PCR product having concentrations 1µl, 2 µl, 4 µl respectively. The fifth lane was of negative control. All bands correspond with 600 bp of standard DNA ladder.
3. In fig. 3 showing band of **Sample A** in second lane of *Brevundimonas diminuta* at 600 bp ladder and sample B in third lane not observing any band. This may be due to no

amplification occurring. This result confirms that 0.2 µm filter is valid for its use in filtration process.

CONCLUSION

Membrane filtration has become a key element for use in the sterilization of fluids (both gases and liquids) during aseptic processing of pharmaceutical solutions. Sterilizing filtration is the process of removing all microorganisms, excluding viruses, from a fluid stream. A sterilizing grade filter must remove all microorganisms present in a fluid stream without adversely affecting product quality. This technical report is intended to provide a systematic Approach to selecting and validating the most appropriate filter for a sterilizing filtration application. Early, careful screening of potential filter types and configurations can result in fewer technical and Regulatory problems, fewer delays, more efficient product processing and greater sterility assurance.

Until the late 1960s, 0.45 µm-rated membranes were considered "sterilizing grade" filters, and were used successfully in the sterilizing filtration of parenteral. Such filters were qualified using 0.6x1 µm *Serratia maracescens*, a standard bacterium for qualifying analytical membranes used for water quality testing (ASTM, 1980). In the mid-1960s, however, Dr. Frances Bowman of the FDA observed a 0.45 µm "sterile-filtered" culture medium to be contaminated with an organism, subsequently shown to penetrate 0.45 µm-rated membranes repeatedly in small numbers at challenge levels above 10^4-10^6 per cm^2 (Bowman et al., 1967). Bowman also observed that the next finer grade commercial membrane (nominally 0.22 µm-rated) effectively retained this organism at similar challenge levels. This 0.3x0.6-0.8 µm contaminant was identified as *Pseudomonas diminuta* (currently reclassified as *Brevundimonas diminuta*), and registered with the American Type Culture Collection (ATCC) as Culture No. 19146. This strain has been accepted widely by filter manufacturers and industry as the standard challenge organism for qualifying sterilizing grade membrane filters (ASTM, 1983; ISO, 1995).

Sterilization is of utmost important from parentral formulation perspective. In filter validation Bacterial challenge test is carried out by conventional method. The major disadvantage of method lies in that it is unable to detect dead microorganisms. *Brevundimonas diminuta* if not viable in pharmaceutical formulation or solutions then various approaches like modifications of process; adjust pH; remove active drug etc; use product for time period challenge organism

viable, change from *Brevundimonas diminuta* use bacteria isolated from formulation, solution or environment are used.

The disadvantage about PCR technique of detecting dead microorganism can be converted in to advantage. By application of PCR procedure for bacterial challenge test.

In the present work by using PCR based microbial detection we can detect the *Brevundimonas diminuta* species though, they are found in not-viable from in pharmaceutical formulation or solution. Therefore, the different approaches mentioned above required to be carried out in ase of non- viable from of *Brevundimonas diminuta* are bypassed.

The study was undertaken to introduce the new method for the microbial testing of the pharmaceuticals, which is based on the Polymerase Chain Reaction hence called as the PCR based microbial testing. On comparison of results it was seen that the PCR based microbial testing has following advantages over conventional microbial testing.

Very rapid : Conventional microbial testing requires the enrichment of the culture but in case of PCR the enrichment of culture is not required hence the time is saved. Again as the incubation of the culture is not required in case of PCR . There are PCR thermal cyclers available which can amplify the 10 to 3000 samples at a time hence no need of repetitively preparing the different media, sterilization of the media and performing the biochemical tests.

Cost effective: Conventional microbial testing requires the more inventory in the form of different equipments e.g. Autoclave, Hot air oven, Incubator etc. and different media for the culturing of the microorganisms, Petri plates, biochemical reagent kits which are different for different microorganisms but in case of PCR based microbial testing all these equipments are not required. The manpower required is also less in case of PCR because 20-3000 samples can be subjected to the PCR based microbial testing at a time hence the labor cost is also less in case of PCR.

Reduced handling of the pathogens: Handling of pathogenic microorganisms is less in case of PCR based microbial testing because few micro liters of the sample is required and we are not enriching the culture but in case of conventional microbial testing we are required to enrich the culture.

Very less space is required: PCR based microbial testing requires less space as compared to the conventional microbial testing.

Simple: Conventional microbial testing requires the change of reagents, media, chemicals for microbial testing of the different microorganisms but in case of PCR same protocol is followed for the different microorganisms, only change required is the change of primer which is specific for the specific microorganisms hence the PCR based microbial testing is simple as compared to the conventional microbial testing which is very tedious.

Sensitive: For PCR based microbial testing the presence of a single DNA, even one single stranded DNA in the sample is sufficient. The sample requirement in case of PCR is 20-100microlitre but in case of conventional microbial testing the sample required is more.

Selective: The PCR based microbial testing is very very selective because only that sequence will get amplified for which the primer is added, but in case of conventional microbial testing the selectivity is less.

Time required is less: Conventional microbial testing requires about three to four days to confirm the presence of *Pseudomonas aeruginosa* but in case of PCR based microbial testing the time required is very less i.e. For the extraction of DNA from the sample, five hours are required, for PCR thermal cycling three hours are required and for the agarose gel electrophoresis two hours are required. Thus it means that we get the confirm result within nine days hence the time required for the PCR based microbial testing is very less.Sometimes due to the incomplete sterilization the microorganism get injured but not completely killed and in case of conventional microbial testing during incubation in the media for the prescribed period of time the microorganism recovers from the injury but do not show the growth and hence false negative result is obtained but in case of PCR based microbial testing no need of incubation and we are concerned with the DNA of that microorganism only hence this disadvantage is absent in case of PCR based microbial testing.

Although the PCR based microbial testing detects the dead microorganism we can use it as a master check technique for the presence of particular microorganism previously i.e. it give the footprints of the presence of microorganism previously and during that microorganism stay it might have released the toxins (exotoxins and endotoxins).

Hence the conclusion is

- By conventional microbial method false positive result may be possible as it can not detect dead organism, but PCR can be detect even dead organism and this is biggest advantage.
- There is a need of fast and accurate method to screen microbes from pharmaceuticals; we have developed PCR based method for quicker and selective microbial testing.
- PCR analysis is selective, simple and cost effective.
- Quantitative analysis will be able to calculate microbial load, which is usually expressed as colony forming units in conventional microbial testing methods.

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