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RESEARCH ARTICLE

EVALUATION AND MICROBIOLOGICAL INVESTIGATIONS OF RADIATION STERILIZATION OF RE-USABLE POLYSULFONE MEMBRANE

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Abstract

One of the most important points in the manufacture of healthcare products is the production of a sterile product with specified (SAL). Healthcare product manufacturers strive to provide safe and sterile products by validating and controlling manufacturing procedures. At present, (SAL) of 10^{-6} is generally accepted for pharmacopoeia and (FDA). The validation studies can be designed in particular for different types of products. Each product needs distinct protocol for bioburden determination, dose mapping and sterility testing. For microbiological tests, culture condition should be selected in both cases of the bioburden and sterility testing. Bioburden is used to describe the population of viable microorganisms present on or in a product and/or a sterile barrier system. Bioburden estimations are used to indicate possible problems in the production process that can lead to inadequate sterilization, calculate the necessary dose for effective sterilization, and to monitor product to ensure adequate dosing. This study presents some results and practical solutions chosen to perform a sterilization validation, compliant with EN ISO Standards, Pharmacopeia and FDA regulations. In this study gamma radiation was selected to sterilize Polysulfone fibers (Allmed PS, SafeFlow and/or POLYPURE filter). Gamma sterilization validation was performed using method 1 in accordance to EN ISO 11137-1, and EN ISO 11137-2 to achieve (SAL) 10^{-6}. Protocol of gamma sterilization validation was achieved.

Introduction:-

In the world of medical devices, "sterilization" is defined as a validated process used to render product free from viable microorganisms. Terminal sterilization is defined as the "process whereby product is sterilized within its sterile barrier system[(1)]**.**Terminal sterilization is a safe and effective approach to manufacture sterile combination products. Combination products have unique material compatibility challenges that must be addressed to ensure successful validation of the sterilization process at a reasonable cost[(2)]**.**

Sterilization is an essential step in the process of producing sterile medical devices. To guarantee sterility, the process of sterilization must be validated. Because there is no direct way to measure sterility, the techniques applied to validate the sterilization process are based on statistical principles [(3)]. Regulatory authorities like EMA and FDA have published guidelines relating to process validation. The purpose of process validation is to ensure varied inputs lead to consistent and high quality outputs. Process validation is an ongoing process that must be frequently adapted as manufacturing feedback is gathered. End-to-end validation of production processes is essential in determining product quality because quality cannot always be determined by finished-product inspection. Process validation can be broken down into 3 steps: process design, process qualification, and continued process verification [(4)].

The number of agents capable of sterilizing product or material without adversely or deleteriously affecting product quality or material integrity is few. There is no singular sterilization method that is compatible with all healthcare products including drugs, polymers, devices, and materials, because of the severity of a process to meet the sterilization criteria and definition[5]. Gamma radiation is an established sterilization method for medical devices and one which is particularly suited to plastics and polymers including Polysulfone and Polycarbonate. A more recent application has been in the sterilization of single-use disposable components. The technology has an advantage over other types of sterilization in that it is capable of deep penetration, although for surface level sterilization, alternative processes such as electron beams are faster[6].

Radiation sterilization has now become a commonly used method for sterilization of several active ingredients in drugs or drug delivery systems containing these substances. In this context, many applications have been performed on the human products that are required to be sterile, as well as on pharmaceutical products prepared to be developed. The new drug delivery systems designed to deliver the medication to the target tissue or organ, such as microspheres, nanospheres, microemulsion, and liposomal systems, have been sterilized by gamma (γ) and beta (β) rays, and more recently, by e-beam sterilization [7]**.**

Gamma sterilization validation Method 1 includes six stages below that shall be followed. Stage 1, Selection of SAL and obtaining samples of product where sterility assurance level 10^{-6} should be used for medical devices Stage 2, Determining average bioburden from samples collected from three different batches, Stage 3, Obtaining verification dose on Bioburden and the required SAL 10^{-2}, Stage 4, Performing verification dose experiment in which 100 product items from the single batch of product have been selected, these product items should be irradiated at the verification dose; then sterility test should performed for 100 products items, Stage 5, Interpretation of results and stage 6, Establish sterilization dose[8].

Bioburden is defined as the number of bacteria living on a surface that has not been sterilized. Bioburden testing is the enumeration and microbial characterization of the population of viable microorganisms on or in a medical device, component, raw material, or package. The term is most often used in the context of bioburden testing, also known as microbial limit testing, which is performed on pharmaceutical products and medical products for quality control purposes[9].An extensive study of the radiation resistance of microbial species constituting the bioburden of a number of different medical devices obtained. A standard protocol for determining radiation resistance was used and validated. The overall distribution of radiation resistance among the isolates was considered to be similar to that forming the "Standard Distribution of Resistance" (SDR) included in the EN ISO 11137- 2. For a number of years, the establishment of an appropriate radiation sterilization dose required to be used for a large range of the medical devices sterilized by ionizing radiation has been based on verification that the radiation resistance of the natural bioburden found on the device does not exceed that of a standard distribution of radiation resistance. Appropriate tables for both the verification dose and sterilization dose, based on bioburden numbers, have been supplied in the EN ISO Standard 11137 [10].

Bulk material sterilization is a process in which a treatment is applied to entire batch in order to decrease its bioburden until a sterility assurance level (SAL) for that specific product is obtained. Validation of the irradiation sterilization of medical devices is regulated [11]**.** Sterilization is a term referring to any process that eliminates (removes) or kills all forms of microbial life, including transmissible agents such as fungi, bacteria, viruses, and spore forms ([12])**.**

Materials and methods:-

Materials:-

Polysulfone high flux membrane (PS, POLYPURE, and/ or SafeFlow of Allmed). Media used in this study were, Thioglycollate Broth, Tryptic Soy Agar (TSA), Tryptic Soya Broth (TSB), supplied by Oxoid, USP. Analytical profile index was used, supplied by bioMérieux.

Validation testing method and strategy:-

Recovery factor (correction factor) determination of Polysulfone filter

Inoculation method was used and artificial bioburden was created because of the bioburden limit of this product is very low [13] by inoculating sterile product with known numbers of spores of (*Bacillus subtilis* ATCC 9372)[14]. The samples were prepared as 5 sterile samples of Polysulfone (POLYPURE and/or SafeFlow) were used during the recovery factor determination. Each sterile sample was inoculated with 100 CFU of (*Bacillus subtilis* ATCC 9372) on the product. Each inoculated product was dried under laminar air flow. Bioburden was determined by elution, followed by membrane filtration and incubation of the membranes on (TSA) medium with subsequent enumeration of the colonies that developed. Recovery factor was determined according to the following equation[11]:

Recovery factor = No of inoculated organisms/ Mean No of spores removed

Bioburden Validation of Polysulfone filter

Bioburden validation was performed, samples were collected from three batches, and thirty (30) non-sterile samples were tested according to requirements of EN ISO 11737-1[11]**.**Microorganisms were isolated and identified. Average of Bioburden samples were calculated to select the applied sterilization dose [15] during gamma sterilization validation. Bioburden tests involve the removal, culture, and enumeration of viable organisms, three general steps that all have a degree of variability [16]**.**Sterile Peptone Water was used as a diluents, diluents should flow through the Polysulfone membrane back into a conical flask, the diluent from the pooling vessel was poured through a bacterial filter, the entire filter was transferred to an appropriately sterile TSA growth medium and incubated at 32.5 ± 2.5^{o}C for 3-5 days, the total number of individual colonies growing on each plate were recorded, the average number of CFU/ sample was estimated as follows:

Average CFU/unit = Total colony count for all units/Number of sample units tested

The result is then expressed as the bioburden estimate, which represents the average of detected bioburden count multiplied by the correction factor.

Identification of Isolated Microorganisms by Gram Staining

Colonies were examined morphologically as shape, size color and microscopically as Gram stain reaction, and the presence or absence of spores. Identification was performed in accordance to the Keys of Bergey's Manual Determinative Bacteriology[17], and Bergey's Manual of Systematic Bacteriology[18], and Cowan and Steel's Manual for the Identification of Medical Bacteria[19].

Identification of isolated microorganisms by analytical profile index (API)

Catalase test was performed for Gram positive bacteria (*Cocci*). Identification was performed according to schematic diagram. Catalase test was performed by using 3% hydrogen peroxide H_2O_2[20] and[21].

Bacterial isolates were identified according to the aforementioned schematic diagram using API. Analytical profile index API STAPH (ID 32 STAPH) was used. API ID 32 Staph (BioMérieux) system was used for Gram-positive cocci as well as other catalase-positive organisms[22].Bacterial isolates were identified according to the aforementioned schematic diagram using API. Analytical profile index API 50 CHL (BioMérieux) was used to identify bacterial isolates with negative results of Catalase test [23]. API *Coryne*(BioMérieux) was used to identify bacterial isolates with positive result of Catalase test (*Bacilli*).

Preparation of Test pieces:-

Isolated taxa were cultivated on TSA agar plate and incubated at 37 °C for 3-5 days. The developed colonies scraped off and suspended in 10% (w/v) horse serum broth, the dense suspension was carefully mixed in sterile porcelain mortar and distributed as separated spots each of 20 µl on sterile polyethylene sheet. The droplets were left to dry at the ambient room temperature on a laminar air flow (LAF). The dried spots were covered with another sterile polyethylene sheet. The two layers were sealed by heating in equal squares each containing one spot [24].

Irradiation of test pieces (Resistant study):-
Test pieces in triplicates were irradiated using Co^{60} gamma source, the radiation doses of 2, 4, 6, 8, 10, and 12 kGy were applied. The number of CFU/ml prior to irradiation (control), as well as the number of survivors after exposure to different doses were determined on TSA agar[25].

Viable count and D-value determination :-
The irradiated test pieces along with the control (non-irradiated) were aseptically removed from their Polyethylene bags, and transferred into 10 ml sterile isotonic saline solution, vigorously shaken using a vortex mixer. Then, ten-fold serial dilution was prepared; 0.1 ml of each appropriate dilution was plated onto sterile TSA agar medium. The plates were incubated at 37 °C for 24 hours, the count of survived colonies as well as the initial was determined and dose response curve was plotted. The sub-lethal dose and D_{10} value of each strain were also calculated [26].

Determination of the verification dose:-
The verification dose has been determined using the average bioburden results. The verification dose is calculated from the overall average bioburden using Table B.1 in ISO 11137-2[8]. By calculating the average bioburden results, the average would be 1.36. Accordingly, the verification dose would be 3.4 kGy±10% for Polysulfone filter as the minimum dose at SAL 10^{-2}. Process control and process capability were calculated.

Verification dose experiment:-
One hundred product units are selected from each type of dialyzers from the same batches as the bioburden test, and irradiated at an average dose. Referring to dose mapping results, samples were irradiated in the most uniform gamma field in the irradiation tote in NCRRT. Irradiated samples were tested for sterility using Thioglycolate medium and incubated at 32.5°C ± 2.5°C for 14 days [27].

Sterility test (Dose verification of gamma radiation):-
Representative samples (n=100) were randomly taken from the most challenge batch, the dose in (kGy) for a SAL of 10^{-2} was obtained from table 5 in ISO 11137-2[8] and in accordance to the bioburden estimated, 100 units of product were irradiated at (SAL) of 10^{-2}. Date of irradiation, the average dose, maximum, and minimum dose should be recorded. Flasks containing the sterility test media, the negative test control, the positive test control and the growth promotion test were incubated at 32.5°C ± 2.5°C, for 14 days and the number of positive tests of sterility were recorded. The 100 flasks of media and product were checked daily. A product positive/ negative sterility test is defined by the presence/ absence of microorganisms growth (turbidity of liquid culture medium) confirmed by isolation in solid culture medium, after the 14 days of incubation. If the entire product is used and verification is accepted, the sterilization dose for the product was obtained from Table 5 in EN ISO 11137-2 using the closest tabulated average bioburden that is greater than or equal to the calculated average Bioburden and read the dose necessary to achieve the desired SAL.

Sterilization dose selection (minimum dose limit):-
Dose setting method 1 has been followed to select the sterilization dose. The sterility assurance level is 10^{-6} as dealing with a medical product for use in vivo.

Results:-

Recovery factor is numerical value applied to compensate incomplete removal from product and/or culture of microorganisms in this study recovery factor determination was conducted in to compensate in complete removal of microorganisms from the product during filtration and extraction in accordance with EN ISO 11737-1[11].

Recovery Factor = 100/100=1

Determination of a population of microorganisms on products has been calculated in order to compensate incomplete removal of microorganisms from Polysulfone filter. Because of the bioburden limit is very low, artificial Bioburden was created by inoculating a known number of a selected microorganism on product in order to establish recovery efficiency. Each sterile sample was inoculated with 100 bacterial spores of (*B. subtilis* ATCC 9372).

The average bioburden observed on 30 samples (collected from three batches) of Polysufone filter was found as 1.2 CFU obtained from Table 2. The maximum Bioburden level of these 30 samples was 2 CFU/device. The minimum bioburden level was (0). Standard division was 0.76.

Bioburden of Polysufone was monitored over one year to make sure the consistency and reliability of bioburden validation. Average of Bioburden over one year was 0.44 CFU as shown Figure 1. Process control and process capability were calculated. Results were normally distributed (p-value was greater than 0.005). Process was within control and capable (capability index is greater than 1) obtained from figure 03, 04 and 05

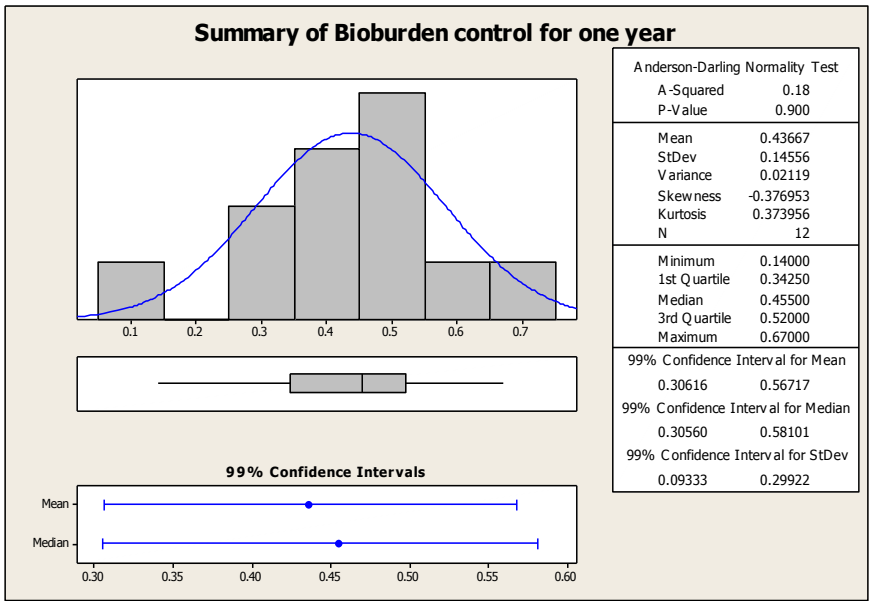

Figure (1) Summary of statistical analysis Bioburden over one year.

Colonies were isolated and identified by gram staining and API. Isolated colonies were identified as gram positive. One taxon was gram positive cocci; and three colonies were gram positive bacilli. Catalsae test was perofomed for 4 isolated taxon to start idetifcation by API. Cataase test was perfomred by using 3% hydrogen peroxide H_2O_2 all the isolates were catalase positive.

Isoalted colonines were identifed by using API STAPH, API 50 CHL and API **coryne** repectively. Identifcatio of isolated colonies obtained from Table 1, 2, 3 and 4.

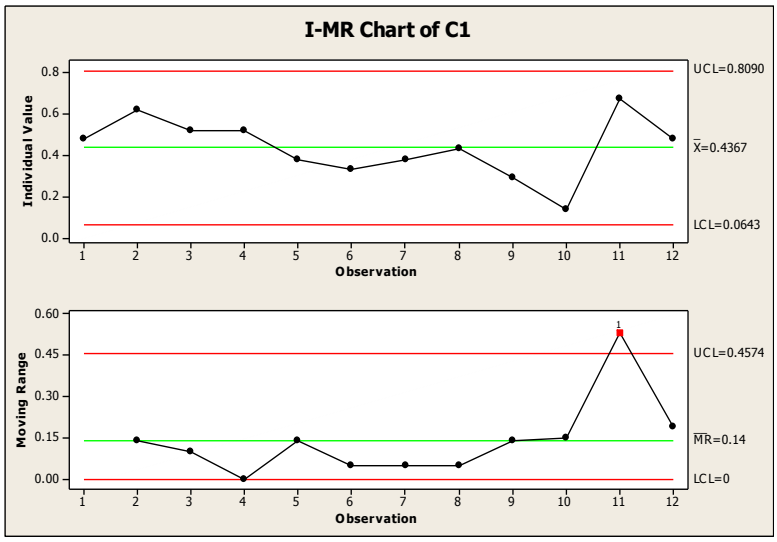

Figure (2) Control chart of Bioburden over one year.

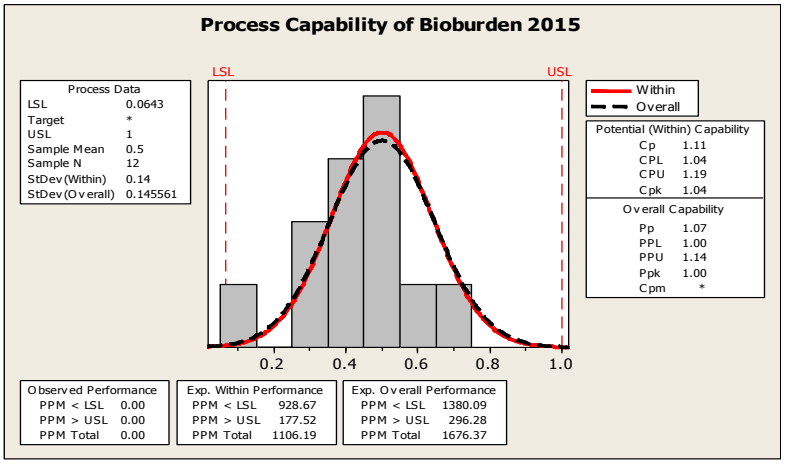

Figure (3) process capability index Cpk

The strains of *S. aureus Lactobacillus delbrueckii Cellulosimicrobium cellulans* and *Leifsonia aquatic* were exposed to different doses. Radiation ranged from 2-12 kGy in 2 kGy interval. Figure (4, 5,6 and 7) show the effect of different doses of irradiation on *S. aureus Lact. delbrueckii Cellulosimicrobium cellulans* and *L.aquatic respectively*. The data showed that the number of viable cell decreased exponentially with increasing the irradiation dose. The D_{10} values were 1.8, 2.0, 2.7 and 2 kGy, and the sub lethal dose was found to be 10 kGy for *S. aureus Lact. delbrueckii Cellulosimicrobium cellulans* and *L.aquatic respectively*.

Table (1): identification of isolated taxon (1) by API *Staph*

GOOD IDENTIFICATION	
Strip	API STAPH V4.1
Profile	6 7 3 6 1 5 3
Note	POSSIBILITY OF Staph.intermedius IF OF VETERINARY ORIGIN

Significant taxa	% ID	T	Tests against			
Staphylococcus aureus	97.8	1.0				

Next taxon	% ID	T	Tests against			
Staphylococcus simulans	1.0	0.74	MAL 11%			

Complementary test(s)	YELLOW	dTURANOSE		
Staphylococcus aureus	+(-)	+(-)		
Staphylococcus intermedius	-	-		

Table (2): identification of isolated taxon (2) by API 50 CHL\V5.1

GOOD IDENTIFICATION	
Strip	API 50 CHL V5.1
Profile	----------+ +----------
Note	

Significant taxa	% ID	T	Tests against			
Lactobacillus delbrueckii ssp delbrueckii	92.4	0.34	GLU 98%	FRU 99%	MNE 81%	ARB 10%
			ESC 24%	MAL 75%	SAC 87%	

Next taxon	% ID	T	Tests against			
Leuconostoc mesenteroides ssp cremoris	6.5	0.12	GAL 90%	GLU 90%	NAG 90%	ARB 0%
			ESC 0%			

Table (3): identification of isolated taxon (3) by API Coryne

EXCELLENT IDENTIFICATION	
Strip	API CORYNE V3.0
Profile	7 5 7 2 7 2 7
Note	POSSIBILITY OF Oerskovia turbata

Significant taxa	% ID	T	Tests against			
Cellulosimicrobium cellulans	99.9	1.0				

Next taxon	% ID	T	Tests against			
Cellulomonas spp/Microbacterium spp	0.1	0.51	PAL 22%	GEL 25%	RIB 22%	GLYG 20%

Complementary test(s)	42°C	NaCl 6%		
Oerskovia turbata	-	-		
Cellulosimicrobium cellulans	+	+		

Table (04): identification of isolated taxon (4) by API Coryne

VERY GOOD IDENTIFICATION	
Strip	API CORYNE V3.0
Profile	2 4 7 2 0 0 4
Note	

Significant taxa	% ID	T	Tests against			
Microbacterium spp/Leifsonia aquatica	99.0	0.98				

Next taxon	% ID	T	Tests against			
Arthrobacter spp	0.9	0.76	ßNAG 14%			

Complementary test(s)	CASEINhyd.	OF/F		
Leifsonia aquatica	-	-		
Microbacterium spp	+(-)	+		

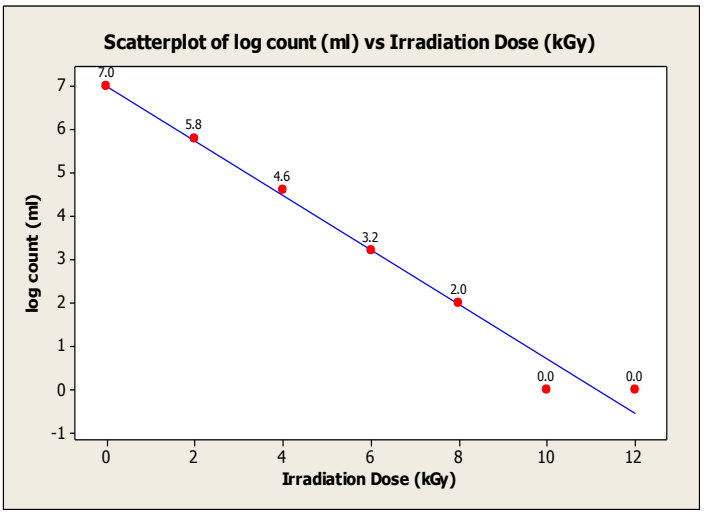

Figure (4) Scatterplot of effect of Irradiation dose on S.aureus

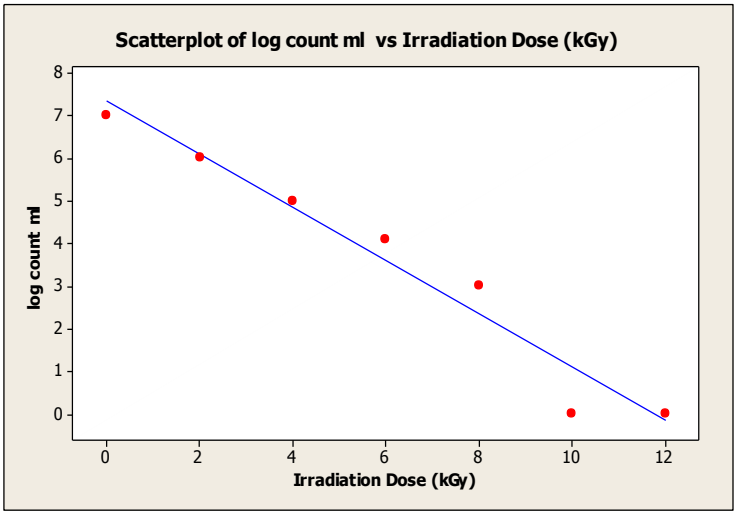

Figure (5) Scatterplot of effect of Irradiation dose on *Lact. Delbrueckii*

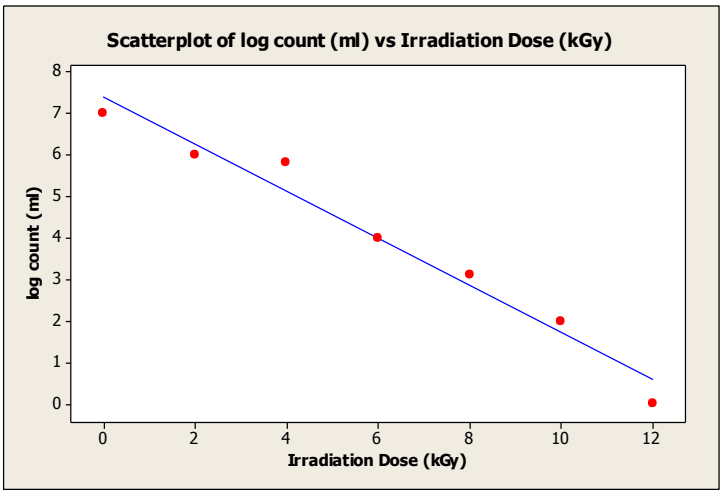

Figure (6) Scatterplot of effect of Irradiation dose on *C. cellulans*

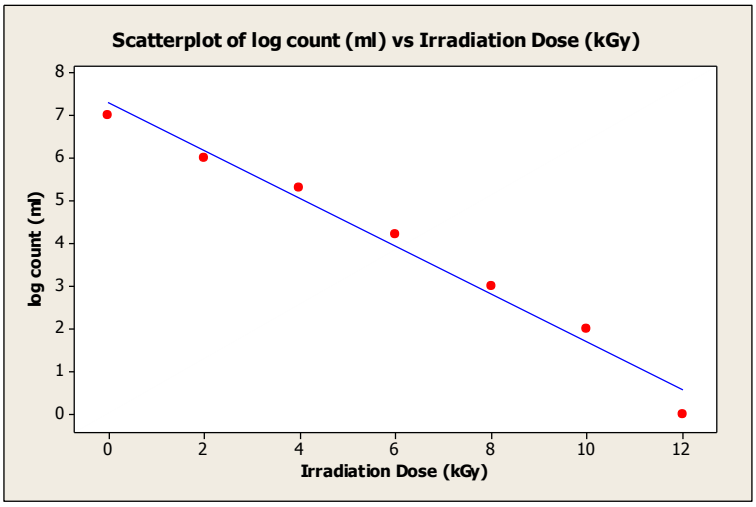

Figure (7) Scatterplot of effect of Irradiation dose on *Leifsonia aquatic*

For estimation of the verefication dose, 100 samples of Polysulfone membranes were selected randomly from batch used in Bioburden experiment and sterilized at 3.4 kGy (Verification dose 10^{-2}) to have final sterility assurance level 10^{-6} (SAL 10^{-6}). Sterility test of 100 irradiated samples were performed. Results showed that non of the examined filters was non sterile. The maximum and minimum dose was recorded and showed in Table (10), the data were obtained from 18 film of dosimeter. The minimum and maximum doses were reported to 3.25 and 3.65 respectively within the specified limits (3.4 kGy +10% = 3.74 kGy). Process was control and capable (Cpk greater than one). If no more than two positive tests of sterility are obtained from the 100 tests carried out, verefication dose is accepted, The verifcation dose was accepted, and the final applied dose (sterilization dose is 15 kGy) [(8)].

Table (05): Readings of 18 dosimeters distributed for verification dose

Film No.	Dose kGy	Film No.	Dose kGy
1.	3.53	**10.**	3.54
2.	3.25	**11.**	3.49
3.	3.54	**12.**	3.59
4.	3.59	**13.**	3.63
5.	3.35	**14.**	3.42
6.	3.52	**15.**	3.49
7.	3.56	**16.**	3.61
8.	3.36	**17.**	3.32
9.	3.59	**18.**	3.64
Average	3.50	**Minimum**	3.64
Maximum	3.25	**STDEV**	0.11

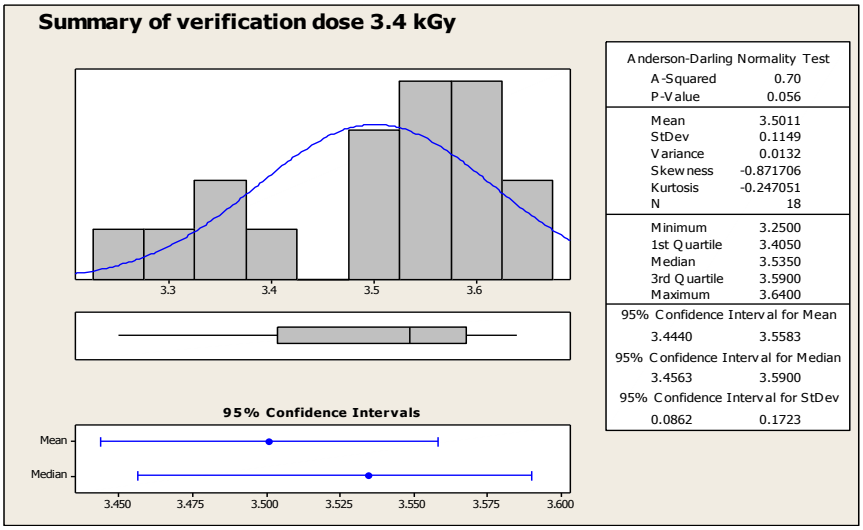

Figure (8) summary of statistical analysis of verification dose (dosimetry)

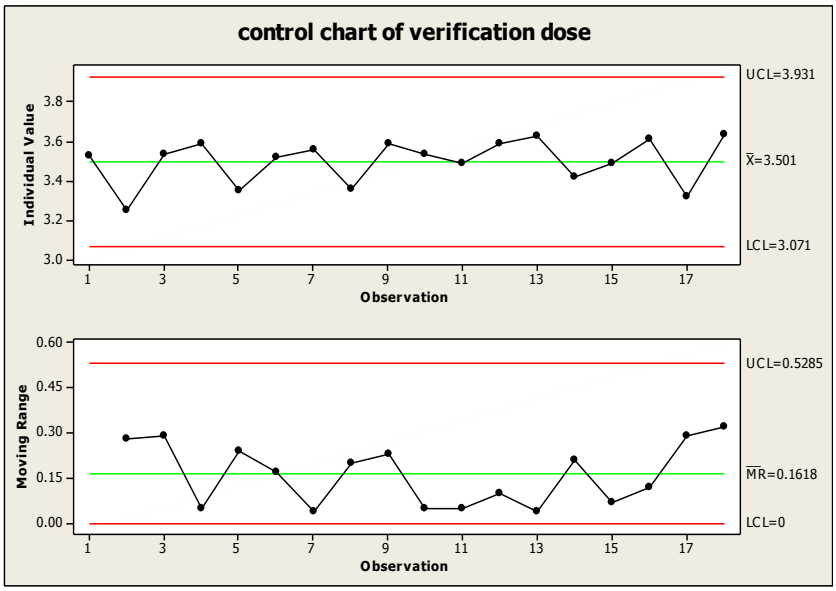

Figure (9) control chart of verification dose (dosimetry)

Conclusion:-

The resutls showed that the sterilization of Polysulfone membrane was aceived with steriltiy assurance level 10^{-6} accoring to the requierments of EN ISO 11137-series, FDA guideline for sterile product, and the United Stata Pharmacopeia (USP) based on the results of Bioburden, Verifcation dose, and steriltiy test (Method 1). The selected dose was 15 kGy. Peromfrnace of Bacterial and Endotxin Polysulfone fitler was achived with bacterial log reduction value (LRV=7), and Endotoxin reduction value (LRV=3.9).

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