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Sterilisation of aseptic drug by sterile filtration: Microbiology validation by microbiology challenge test

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ABSTRACT

Aseptic drugs are often difficult to sterilize due to their inherent sensibility to heat-induced degradation. According to Good Manufacturing Practices (GMP), if a terminal sterilization is not possible, filtration through a bacteria retentive filter or aseptic processing is used. Filtration of liquids through 0.2 µm diameter filters is a common method for removing microorganisms from the drug heat-sensitive solutions to obtain sterile filtrate. Actually, final filtration with a 0.2 µm diameter filter is the simplest last unit operations in the process; however, it is a critical step for successful manufacturing. Filter clogging can occur primarily because of molecular aggregation and can result in the deterioration of product quality and longer processing times. This shows the aim of the validation of the sterile filtration which requires many evaluations including a bacterial challenge.

Keywords: Aseptic process; Validation; Bacterial challenge; sterile filtration; sterilization.

INTRODUCTION

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 subvisible particulates from parenteral preparations, particularly solutions introduced intravenously, was found to be important [1].

When looking at filtration as an overall technique which ensures quality for pharmaceuticals products, there is only one clearly defined and accepted level of rating: the sterile filtration [2]. According to the Food and Drug Administration (FDA, USA) and the United States Pharmacopoeia (USP), sterile filtration is defined as a nominal rating of 0.2 µm and 0.22 µm respectively to produce sterile effluent [3-7]. Thus, the obvious objective of a sterile filtration step is the removal of any viable microorganism that may be present in the bulk product. This is typically accomplished by the use of "Sterilizing Grade" membrane filters, defined in the FDA, as those capable of totally retaining a challenge level of 10^7 CFU/cm^2 [4]. The FDA currently accepts *Brevundimonas Diminuta* at this challenge level as a worst case model in validation, but it is also acceptable to use natural flora. Actually , until the late 1960s, 0.45 m-rated membranes were considered "sterilizing grade" filters, and were used successfully in the sterilizing filtration of parenterals. Such filters were qualified using 0.6x1 µm *Serratia marcescens*, a standard

bacterium for qualifying analytical membranes used for water quality testing. 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 [9].

Bowman also observed that the next finer grade commercial membrane (nominally 0.22 m-rated) effectively retained this organism at similar challenge levels. This 0.3 X 0.6-0.8 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 [9-13]. Following the broad acceptance of *B. diminuta*, FDA incorporated demonstration of its retention in the definition of a sterilizing filter [14].

The use of this microorganism provides several advantages [15]:

-Originally a process stream isolate, it is therefore a realistic potential problem organism;

-Generally regarded as nonpathogenic to humans, ordinary microbiology laboratories can use it without major biohazard concerns;

-It can be consistently cultured under controlled conditions to yield very small, monodispersed cells with a narrow size distribution. These can penetrate 0.45 m filters reproducibly in small numbers at high challenge levels, thus representing a potential worst-case challenge.

The validation of a sterile filter means that the function of the filter has been checked with different and correlated challenge tests, and that these parameters are available for both the filter manufacturer and the filter user, in order to perform an integrity test [16]. Validation of a filter briefly means that parameters are measured and given as a proof of the desired function of the filter, which must be linked to how the filter works in reality [2,13,17]. The most important factors that impact on the choice of filter and filter systems are the dirt capacity of the filter, the physical and chemical compatibilities of the filter, all together with the design and the function of the process.

A sterile filter is generally intended for the elimination of harmful particles from a fluid. Simultaneously, the filter system must be inert; the filter system should not add or remove anything from the fluid, even though it may not be regarded as a contaminant at first glance. This is why all the different parts of a filter system in contact with the process flow must be tested [18].The filter manufacturer performs such tests and sometimes these tests are also performed by the filter user. Particle release, extractable substances, and physical as well as chemical compatibilities, all together with absorption of critical substances are some of the factors that are usually investigated. Toxicity tests, bacterial challenge tests and physical integrity tests constitute others important tests that are performed [19, 20].

The aim of this work was to accomplish a part of validation, which concerned especially the bacterial challenge test. The aim of bacterial retention validation studies was to have documented evidence demonstrating that the filtration process generated a sterile effluent; and reliably removed a high level of a standard bacterium or relevant bioburden isolate suspended within our product under simulated worst case processing conditions. Factors potentially affecting microbial retention include filter type (structure, base polymer, surface modification chemistry, pore size distribution, thickness), fluid components (formulation, surfactants, additives), fluid properties (pH, viscosity, osmolarity, ionic strength), process conditions (temperature, differential pressure, flow rate, process time, re-use, sterilization and re-sterilization) and the specific characteristics of the actual bioburden in the product [21].

EXPERIMENTAL SECTION

The first step was to determine the most suitable method for performing a liquid bacterial challenge test for product and process specific filter validation. The viability test method was based on the principles described in Parenteral Drug Association (PDA) Technical Report n°26 [22] which are:

-The test organism's viability should be verified by direct inoculation into the carrier fluid (product or stimulant);

-The micro-organism for viability testing has grown in the same way as that used for bacterial challenge testing, in order to preserve its morphological and physiological characteristics;

-The test exposure time should equal or exceed the actual process filtration time. The test sample can be considered non-bactericidal if no more than a one log reduction in count is noted, after the exposure time. If a reduction in

microbiological concentration of more than one log is noted, the product should be considered bactericidal and an alternative testing methodology considered.

-The challenge organism may survive in the product under normal processing conditions, but not for total processing time. This is considered a moderately bactericidal product. In this case, the product can be inoculated directly with the challenge organism for the viability duration (a minimum time period of 30 minutes).This challenge should be done after preconditioning the filter with the product under process conditions.

To qualify as sterilising grade for a filter, it needs to provide a sterile filtrate when challenged with *Brevundimonas Diminuta* bacteria at minimum challenge level of 1 X 10^7 CFU/cm^2 of filter area.

In our case, we choose to study the test product made from Tiemonium methyl sulfate and which is sterilized by aseptic liquid filtration using N66 filter cartridge (SLK7001 NFP) (Pall). In our case, it could not be possible to work with the actual test sample, due to its toxicity, abuse potential, limited supply or bactericidal activity. The simulant fluid had to match the product as closely as possible terms of its physical and chemical characteristics, without adversely affecting the challenge micro-organism.

1. Viability study

1.1 Viability Study

Historically, P. diminuta, recently reclassified to *Brevundimonas diminuta* (ATCC 19146), has been selected as the microorganism of choice. *Brevundimonas diminuta* has been used to rate sterilising grade filters at a concentration in excess of 10^7 organisms per cm^2 of Effective Filter Area (EFA) with testing carried out following ASTM F838-05 test methodology [17].

It is important to determine if the standard challenge organism is viable in the process fluid for the exposure time required, simulating the expected period of filter use. Where organisms are non-viable, alternative organisms or surrogate process fluids may be recommended.

The microorganism for viability testing *Brevundimonas diminuta* (ATCC 19146) has grown in the same way as that used for bacterial challenge testing, in order to preserve its morphological and physiological characteristics [22]. The test organism's viability was verified by direct inoculation into the tested product at 18°C - 25ºC. Aliquots of the product sample were taken over a time period that exceeded the real process filtration time (T=1 minute, 60 minutes, 120 minutes, etc… until the maximum time). Each aliquot was serially diluted and a Total Viable Aerobic Count (TVAC) was performed and determined using membrane filtration (Pall). The filter membrane was flushed with an additional minimum of 25 mL of 0.9% NaCl (H. Möller GmbH & Co. KG) to remove potential test product residues inhibiting growth of the test organism during incubation. This step was repeated using phosphate buffered saline as a control. The filter membrane was then incubated aerobically for a minimum of 2 days at 30 ± 2°C on Tryptone Soya Agar (TSA) (Biokar).

1.2 Flush studies

Performing bacterial retention testing on bactericidal products makes it more difficult to answer both questions relating to validation: what effect does the product have on the filter, and what effect does the product have on flora within the product. Bacterial retention testing performed on a bactericidal formulation or under challenge conditions adverse to microbial viability (e.g., elevated temperature) may not produce valid results.

To overcome these obstacles, an alternate testing methodology is required. This may involve modification of the challenge fluid or challenge conditions or a combination of the two.

To evaluate the potential effect of the product/process on the filter, the filter may be preconditioned with the product under actual processing conditions, including flow rate, pressure, temperature and time. This preconditioning may be performed by recirculating the product through the test filter in a closed loop system, or by a single pass through the test filter [23].

1.2.1 Recovery Filter Flush Study

The recovery filter membrane was situated downstream of the test filter during the bacterial challenge test and recovered the test organism if penetration had occurred through the test filter, under process specific testing conditions. One recovery membrane was left in contact with the test product for the maximum processing time, and then flushed with 1000 mL of water for injection, to remove potential bactericidal residues inhibiting growth of the test organism during incubation. The recovery membrane was then inoculated with the test organism and placed into TSA. The TVAC was compared to a control.

1.2.2 Test Filter Flush Study

The test filter membrane was situated upstream of the recovery filter during the bacterial challenge test. If the process product was bactericidal, then the bacterial challenge study consisted of a product re-circulation before the recovery membranes were assembled. It was critical that bactericidal product residues were removed from the system upstream before the test organism and recovery membranes were introduced. The test filter membrane was left in contact with the test product for the maximum processing time, and then flushed with 1000 mL of water for injection to remove potential bactericidal residues. The last 10 mL of the flush was collected and inoculated with the test organism and a TVAC was performed using membrane filtration and compared to a control. The exposed test filter membrane was also inoculated with the test organism and placed into TSA.

Achievement of acceptance criteria is summarized below **(Table 1):**

Table 1. **Acceptance Criteria Met**

Test Reference	Acceptance Criteria	Acceptance Criteria Met
Viability Test Control Sample	Control demonstrates no more than a one log reduction in count **[21].**	YES
Recovery Filter Flush	The TVAC of the test sample must be equal to or greater than 70% of the control sample **[22].**	YES
Test Filter Flush	The TVAC of the test sample must be equal to or greater than 70% of the control sample **[22].**	YES

Abbreviation: *TVAC= Total Viable Aerobic Count.*

2. Bacterial challenge study :

According to the PDA : "The goal of conducting bacterial retention validation studies is to generate data demonstrating that the filtration process will consistently remove high levels of a standard bacterium, or relevant bioburden isolate, suspended within product (or surrogate fluid), under actual process conditions".

The bacterial challenge test serves two major functions. The filter manufacturer uses it to classify filters as sterilizing grade if the filter provides a sterile effluent with a minimum of 10 cells of *B.diminuta* ATCC 19146/cm^2 of effective filter surface area.

Bacterial challenge tests also are required to validate the sterilizing filtration process of a specific product. The filter challenge test must be performed with actual product or, where justified, suitable surrogate fluid.

The Bacterial challenge protocol proceeds as follows:
Three filter membrane lots were included in product bacterial retention validation studies:

-At least, one of the filter membrane batch used for bacterial retention validation had a pre-filtration physical integrity test value at or near the filter production limit;

- Physical integrity was determined prior to challenge testing, using water, product or other dampening fluid for which specifications exist;

-If the test organism was recovered downstream of any filter membrane after the product bacterial challenge, an investigation was performed. If such investigation confirmed penetration of the filter membrane by the test organism and the filter met its integrity test specification, then the applicability of the filter under these process conditions had to be reconsidered.

-

2.1 Preparation of Test Organism

Challenge Organism Selection Criteria:

-The challenge bacteria had to be small enough to challenge the retention of the sterilizing grade filter and simulated the smallest microorganism that could occur in production [24];

-"A sterilising grade filter had to be validated to repeatedly remove viable microorganisms from the process stream producing a sterile effluent" [2] ;

-"*B. diminuta* has grown under standard culture conditions penetrated 0.45 μm-rated membranes in small numbers at high challenge levels (typically > 10^7)"[22].

The micro-organism for bacterial challenge testing (*B. diminuta* ATCC 19146) was prepared from frozen cell paste, in order to preserve its morphological and physiological characteristics. *B. diminuta* American Type Culture Collection (ATCC) 19146 was supplied as a frozen cell paste producing suitable suspensions of *B. diminuta* of approximately 0.3-0.4 μm in diameter by 0.6-1.0 μm in length [25].

2.2 Bacterial Challenge Study

The bacterial challenge test validates the ability of a filter to provide sterile effluent in a specific pharmaceutical liquid. It is also the ultimate compatibility test, because the bacterial challenge simultaneously tests the physical chemical interaction of the liquid product and the filter, under process conditions. Any filter inadequacy caused by this interaction will be detected by the bacterial challenge [26, 27].

The bacterial challenge study was performed using three Ultipor N66 filter membranes (Pall) from three different batch numbers. At least, one lot number of the test filter was made from membrane material, which was at or near to the manufacturing production limit and was, therefore, at minimum specification. The test filter membrane and control filter membrane were "Bubble point" tested pre and post bacterial challenge to confirm correct installation into the disc holder. The test equipment was autoclaved and sterilised at 125 °C for 60 minutes (Lequeux). Pressure gauges were sanitized in 60/40 Isopropyl alcohol (IPA)/Water for a minimum time period of 15 minutes. The test assembly was aseptically assembled in a laminar flow cabinet (Telestar).

For this process, our test product was directly inoculated with the challenge organism at the end of the product exposure at 120 minutes to deliver a minimum challenge level of 10^7 CFU/cm^2 of filter surface area, under simulated processing conditions, including time, temperature, pressure and other critical variables. To minimize product adulteration by the inoculum, the inoculum volume was as low as possible **(Table 2).**

Table 2. **Selection Criteria for Manufacturing Processes and validation test**

Parameters	Process Parameters		Validation Test Parameters
	Routine	Worst case	
Filter part number	2 x SLK7001NFP	2 x SLK7001NFP	FTKNF*
Filtration area (cm²)	1400	1400	12,5
Filtration mode	Constant Pressure Driven	Constant Pressure Driven	Constant Pressure Driven
Total exposure time	7 hours	36 hours	39 hours **
Pressure (mbar)	200	500	500
Product temperature (°C)	18-25	18-25	18-25
Sterilization conditions	Autoclave 122°C for 45 min	Autoclave 122°C for 45 min	Autoclave 125°C for 60 min

Test disc membrane FTKNF was made from the same membrane used to manufacture SLK7001NFP;
*** Due to laboratory shift, the test contact time was extended to 39 hours.*

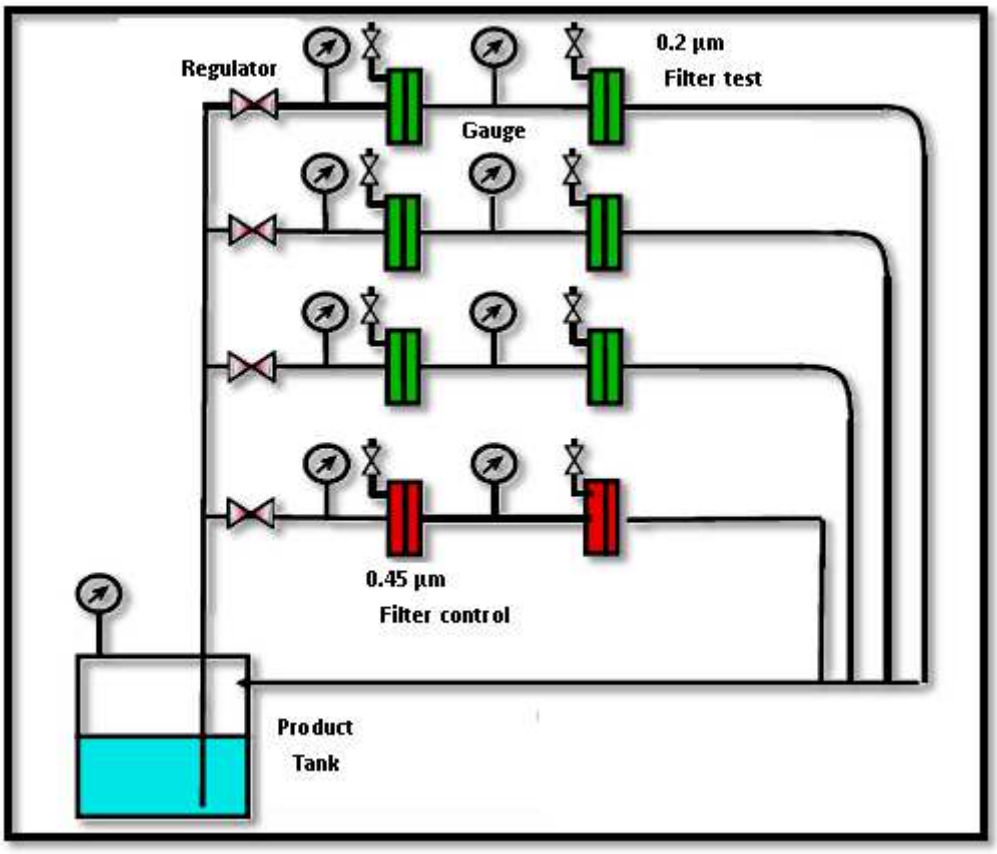

Figure 1: Bacterial Challenge Test Re-circulation

2.3 Downstream Sampling

Challenge effluent analysis was performed by direct passage through a 0.22 μm rated recovery filter membrane installed downstream of the test filter *(Figure 1).* The test filters had to avoid penetration of the test organism and demonstrate no recovery of the test organism. The control filter had to allow penetration of the test organism and demonstrate the recovery of one or more CFU of the test organism. A TVAC of the challenge inoculum was performed at the start and the end of the challenge. The recovery filter membranes, positive controls, and TVAC of the challenge inoculum were incubated aerobically at 30 ± 2 ºC on TSA and read at 7 days [29]. The appropriate flush regimes were used as determined during the viability study.

2.4 Acceptance Criteria :

Achievement of acceptance criteria is summarized below (**Table 3**):

Table 3. Acceptance criteria not met

Acceptance Criteria	Corrective Actions if acceptance criteria not met
Each of the test filters and the control filter passed a prechallenge filter bubble point test.	If the bubble point integrity test prior to the challenge did not meet acceptance criteria, replace the disc with a new disc from the same batch and test again.
Each test filter and control filter was subjected to a challenge of *B. diminuta* (ATCC 19146) at a minimum of 1.0 x 10^7 CFU/cm^2 filter surface area	If bacterial cell paste titre was lower than expected (approximately 1.0 x 10^{10} CFU/ mL) investigate low cell paste titres and repeat bacterial challenge test. If bacterial cell paste titre was satisfactory and the TVAC in the challenge fluid was lower than expected, review viability test data and non-bactericidal or stimulant sample.
The control membrane (0.45 μm pore size) had to allow penetration of one or more colony forming units to verify physical monodispersion of the test organism (Positive Control)	Review viability and flush qualification results for the product and simulant; confirm pore rating of the control membrane by reviewing the bubble point integrity test performed at the start of the challenge.
Each of the test filters and control filter passed a post challenge filter bubble point test.	If the bubble point test at the end of the challenge did not meet acceptance criteria and recovery was observed, repeat the bacterial challenge test.
Defined challenge test parameters had to be met (i.e. maximum flow rate and /or differential pressure, temperature and time period).	If bacterial challenge test parameters were not met, determine the root cause.
The test filter recovery membranes had to show no growth of *B. diminuta*.	Gram stains had to be performed on all microbial growth. Isolates identified as Gram negative rods had to be identified to species level by biochemical identification, if possible. Microorganisms that were identified as *B. diminuta* demonstrated test filter failure. Micro-organisms that were identified as not being *B. diminuta* demonstrated recovery filter contamination. If gross contamination was observed, the presence of *B. diminuta* could be masked, therefore, the challenge study had to be repeated.

Abbreviation: *TVAC= Total Viable Aerobic Count.*

Achievement of acceptance criteria is summarized below (**Table 4**):

Table 4. Acceptance criteria Met

Acceptance criteria	Acceptance criteria Met (Yes/No)
Each of the test filters and control filter passed a prechallenge filter bubble point test	YES
Each test filter and control filter was subjected to a challenge of *B.diminuta* (ATCC 19146) at a minimum of 1.0 X10^7 CFU/cm^2 filter surface area	YES
The control recovery membrane had to allow penetration of one or more colony forming units to verify physical monodispersion of the test organism (Positive control)	YES
Each of the test filters and control filter passed a post challenge filter bubble point test	YES
Defined challenge test parameters had to be met (maximum flow rate end/or differential pressure , temperature and time period)	YES
The test filter recover membranes had to show no growth of *B. diminuta* (ATCC 19146)	YES

RESULTS AND DISCUSSION

1. Viability study :

1.1 Viability study results

The TVAC was calculated by choosing the dilution factor that yielded a bacterial count between 30-300 CFU [30] ; this had then been divided by the volume of the dilution and the lowest average value was reported. The lowest TVAC was 1 CFU at 10^{-4}. Therefore, the minimum detectable count was 1 x 10^4 CFU/mL. Results of the Viability Study Test Results are shown in **Table 5**.

Table 5. Viability Study Test Results

Test Sample	Average TVAC (CFU/mL)				Log Reduction
	1 min	60 min	120 min	Maximum processing time	
Liquid product X	$3{,}20 \times 10^7$	$1{,}68 \times 10^7$	$1{,}17 \times 10^7$	$1{,}06 \times 10^6$	< 1 within 120 min
Control	$3{,}30 \times 10^7$	$3{,}20 \times 10^7$	$3{,}40 \times 10^7$	$2{,}41 \times 10^7$	> 1 across the entire contact time

Abbreviation: *TVAC= Total Viable Aerobic Count.*

Concerning the viability Test Control Sample, the control demonstrates no more than a one log reduction in count

1.2 Recovery Filter Flush Studies

The TVAC of the test sample is equal to the control sample .The test ensure that the specified acceptance criteria is met. Results of the recovery filter flush study are shown in **Table 6**.

Table 6. Recovery Filter Study Test Results

Test Sample	Flush Fluid	Flush Volume (mL)	TVAC (CFU/membrane)	Acceptance Criteria Met? (YES/NO)
Liquid product X	Water for injection product (WFI)	1000	291	YES
Control			295	

Abbreviation: TVAC= Total Viable Aerobic Count.

1.3 Test Filter Flush Studies

Results of the test filter flush studies are shown in Tables 7a and 7b.

Table 7a. Test Filter Study Test Results – Inoculated Flush Fluid

Test Sample	Flush Fluid	Flush Volume (mL)	TVAC (CFU/membrane)	Acceptance Criteria Met? (YES/NO)
Product X	WFI	1000	280	YES
Control			296	

Abbreviation: *TVAC= Total Viable Aerobic Count.*

Table 7b. Test Filter Study Test Results – Exposed Test Filter membrane

Test Sample	Flush Fluid	Flush Volume (mL)	TVAC (CFU/membrane)	Acceptance Criteria Met? (YES/NO)
Product X	WFI	1000	232	YES
Control			287	

Abbreviation: *TVAC= Total Viable Aerobic Count.*

The test filter and recovery filter flushes were within the acceptance criteria.

2. Bacterial Challenge :

The test and control filters were installed into disc holders and dampened with water. These were then bubble point tested to confirm installation and pore rating (**Table 8**).

Table 8. Bubble point results

Filter	QBP * of filter membrane (psi)	Installation Bubble point Test Value (mbar)		
		Min acceptable	Before Challenge	After Challenge
Ultipor N66	50.9	3180	3750	3650
			Pass	**Pass**
Ultipor N66	49.4	3180	3450	3450
			Pass	**Pass**
Ultipor N66	50.2	3180	3650	3700
			Pass	**Pass**
Ultipor N66 Positive control	NA	2000	2200	2250
			Pass	**Pass**

Abbreviations: *QBP= Quantitative Bubble Point.*

**** QBP****: Quantitative Bubble Point (QBP) of the membrane used to manufacture the 0.22 µm sterilising grade filter discs was measured on membrane wet with water.*

The equipment used to conduct the test was the Palltronic (Pall). This equipment is automatic and designed and manufactured in compliance with GAMP standards [28]. The Palltronic system is calibrated at Pall-certified service center.

To run the integrity test, we simply connected the Palltronic to the filtration system and started the program, which conducts the test automatically while the touch screen displays the progress of the test. No operator intervention is required during the test. At the end, the test results are printed by the system as documentary support.

After, the TVAC were performed in the product test that had been inoculated with the test organism typically at the start and the end of the bacterial challenge test exposure; the lowest was used to calculate total challenge (**Table 9**).

Table 9. TVAC results

Filter Part	Total challenge (CFU)	Challenge per filter area (CFU/cm^2)	Recovery
Ultipor N66	3.15 X 10 ^{8}	2.52 X 10 ^{7}	N/D
Ultipor N66	3.15 X 10 ^{8}	2.52 X 10 ^{7}	N/D
Ultipor N66	3.15 X 10 ^{8}	2.52 X 10 ^{7}	N/D
Ultipor N66 Positive control	3.15 X 10 ^{8}	2.52 X 10 ^{7}	TNTC

Abbreviations: *N/D: Not Detected; TNTC: Too Numerous To Count.*

The results of the viability study test demonstrated that the test organism, *B. diminuta* (ATCC 19146) was not viable in the product test for the maximum processing contact time; however the test organism was viable in the tested product at a minimum time period of 120 minutes.

The test organism was directly inoculated in the tested product during the final 120 minutes to deliver a minimum challenge level of 10^7 CFU/cm^2. The test filter and recovery filter flushes were within the acceptance criteria. All filters tested in this study met Forward Flow integrity test specifications, both pre- and post-challenge, and were, therefore, integral. These filters were bubble point tested using 60/40 IPA/water as the dampening fluid, and these values were reported in Table 8. The three 0.2 µm rated "sterilizing grade" filter types from Pall were tested, and the bacterial challenge test results were summarized in Table 9. All three filters tested produced a sterile effluent, when challenged for the maximum processing time with total challenges of 2.52 x 10^7CFU. All three filters produced a sterile effluent.

The bacterial retention validation study generated data demonstrating that the filtration process consistently removed a high level of a standard bacterium (using *B. diminuta),* suspended within the product test under simulated worst case processing conditions. It proves that the production process generated a sterile effluent. These results validated the efficacy of sterile filtration of SLK 7001NFP under the conditions previously described and support the use of functionally qualified 0.2 µm rated filters as sterilizing grade filters in our pharmaceutical operations (Table 10).

Actually, nowadays, concerns have been raised regarding the potential effect of drug product properties and composition on microorganism 'size and/or its ability to be retained by 0.2/0.22 µm rated filters. It is well known that bacterial sizes in laboratory culture are not the same as under industrial process conditions. In the harsh environment of a pharmaceutical process, nutrients can be very limited and many Gram negative microorganisms have been shown to reduce up to 45-75% in cell volume under such nutrient deprivation conditions. In some cases, changes in osmolarity can induce a change in the size of the microorganisms ; for example, *E.coli* have been shown to decrease about 15-20% in size when taken from a 150 mosM NaCl solution to 300 - 500 mosM NaCl . Also, the presence of specific enzymes or antibiotics may induce L-forms that have been known to penetrate filters that usually retain the parental strains [31].

The three different 0.2 µm rated sterilizing grade filter types tested in this study differed widely in many characteristics. For example, the QBP of filter membrane of the three 0.22 µm rated filters tested ranged from 49.4 psi to 50.9 psi, a variation of less than 15%. While a minor portion of this difference is almost certainly attributable to differences in pore morphology and surface chemistry, the range is too large to be solely related to such effects and is indicative of significant differences in pore size distributions (as the bubble point of a filter is considered by some authors as an indicator of the largest set of pores in the filter membrane), and hence, microbial removal performance [25]. In theory, filtration or process fluid parameters that modify the physicochemical properties of the filter membrane or bacteria can impact passage. In other publications, high viscosity has been suggested to decrease retention by increasing processing time [32, 33].

Besides the product bacteria challenge test, tests of extractable/leachable substances and/or particulate releases have to be performed. Extractable measurements and the resulting data are available from filter manufacturers for the individual filters. Each filter used in aseptic processing requires individual process and product related validation efforts. Evidence has to be given that the filter is working under the conditions specified by the user.

The current study, thus, provides additional evidence for the efficacy of functionally qualified 0.22 µm rated filters to consistently produce sterile effluents under similar test conditions that resulted in penetration of 0.2/0.22 µm rated filters.

Table 10. Summary results matrix

COLUMNS	A	B	C	D									E
	PROCESS PARAMETERS			**VALIDATION TEST PARAMETERS**									
PARAMETERS	ROUTINE	WORST CASE	REQUIRED										TEST DEVIATION
Product	X	X	X	X									No
Filter part number	2XSLK7001 NFP	2XSLK7001 NFP	FTKNF	FTKNF									No
Filtration area (cm^2)	1400	1400	12.5	12.5									No
				Line 1			**Line2**			**Line3**			No
Filter batch number	Not applicable	Not applicable		NK1078			NK1079			NK1080			No
Filtered volume (mL)	132000	176000	Not applicable	Not applicable			Not applicable			Not applicable			No
Filtration mode	Constant Pressure Driven	Constant Pressure Driven	Constant Pressure Driven	Constant Pressure Driven									No
Total product exposure time	420 min (7 hours)	2160 min (36 hours)	2340 min (39 hours)	2340 min (39 hours)									No
Challenge time	Not applicable	Not applicable	Final 120 min	Final 120 min									No
Filtration time	420 min (7 hours)	2160 min (36 hours)	2340 min (39 hours)	2340 min (39 hours)									No
Product Exposure phase				**Line1**			**Line2**			**Line 3**			
Time Point (minutes)				T= 1 min	T= 1440 min	T= 2220min	T= 1 min	T= 1440 min	T= 2220min	T= 1 min	T= 1440 min	T= 2220min	
Flow rate (mL/min)	Not applicable	Not applicable	Not applicable	30	39	36	30	36	42	30	36	39	No
Flow rate per unit area (mL/min)/cm^2	Not applicable	Not applicable	Not applicable	2.4	3.1	2.9	2.4	2.9	3.4	2.4	2.9	3.1	No
Pressure (mbar)	200	500	500	500	500	500	500	500	500	500	500	500	No
Product Temperature(°C)	18-25	18-25	18-25	22.2	23.2	23.6	22.2	23.2	23.6	22.2	23.2	23.6	No
Challenge Phase				**Line1**			**Line2**			**Line 3**			
Time Point (minutes)				T= 1 min	T= 60 min	T= 120min	T= 1 min	T= 60 min	T= 120min	T= 1 min	T= 60 min	T= 120min	
Flow rate (ml/min)	Not applicable	Not applicable	Not applicable	24	18	15	24	18	18	24	18	15	No
Flow rate per unit area (mL/min)/cm2	Not applicable	Not applicable	Not applicable	1.9	1.4	1.2	1.9	1.4	1.4	1.9	1.4	1.2	No
Pressure (mbar)	200	500	500	500	500	500	500	500	500	500	500	500	No
Product Temperature(°C)	18-25	18-25	18-25	23.6	23.9	23.9	23.6	23.9	23.9	23.6	23.9	23.9	No

CONCLUSION

In this report, we have documented that the three different 0.22 µm rated sterilizing grade filter types, from the same manufacture, consistently produced sterile effluents under similar test conditions. However , some studies suggests that knowing the native bioburden (both quantitative and qualitative) and the potential effects of the process fluid on those organisms are critical in selecting the correct sterilizing grade for each application, thus, providing effective aseptic processing of drug products [21]. These studies support the use of functionally qualified 0.2 µm rated filters as sterilizing grade filters for applications either for long-term filtration processes, or when bacteria like *B. diminuta* are present in the bioburden.

Product and/or process conditions, under which penetration of 0.2/0.22 µm rated filters have been reported (by the FDA) to have occurred, such as drug solutions that either support growth of bioburden in the product, or that provide minimal growth support of bioburden in product (namely, nutrient deprived solutions), or that contain lipids, may also benefit from the enhanced sterility assurance associated with the use of functionally qualified 0.2 µm rated filters. Actually , there is increasing awareness that the current industry standard for sterilizing filters, namely 0.2/0.22 µm rated filters qualified with *B. diminuta* as per ASTM Method F838-83 (described by the American Society for Testing and Materials) [34] or comparable methodology, does not necessarily guarantee sterility for all bacteria under all conditions. This has led to renewed emphasis on routine bioburden assessment, quantitative as well as qualitative, to support the continued use of 0.2/0.22 µm rated filters. Thus, it has been recommended that, apart from knowing the viable bioburden count (in CFU/mL) in the drug product and/or process, one must also identify and specify the microorganisms present. The hope is that this information can be used to justify the use of 0.2/0.22 µm rated filters on the basis that the bioburden does not contain any microorganisms, which can penetrate the filter more easily than *B. diminuta*, used in process- and product-specific bacterial retention validation studies [35].

We noted that the final selection of filter material and pore size should be based on available microbiological retention data, which then have to be strengthened by process specific validation work [36]. Sole reliance on pore size rating, besides promotion of smaller pore size increasing safety, is inappropriate and often unnecessary. Each individual process requires, through review and tests, to back-up the final decision of which filter configuration and pore size should be used [37]. An overall approach cannot be justified as being safer, when it is not required.

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