A New Direction in Mycoplasma Control

Martha Folmsbee, Morven McAlister and Jerold Martin at Pall Life Sciences discuss some of the challenges involved in the validation of sterilising filters for mycoplasma removal and the importance of the associated process-specific tests compared to the filter manufacturer's standard tests

Mycoplasma are very small bacteria distinguished by the lack of a rigid cell wall, and have been reported as common contaminants of cell cultures and cell culture media (1,2). Presumably, due to their small size and lack of rigidity, they are known to be capable of penetrating 0.2 and 0.22µm rated sterilising grade filters, and even some 0.1µm rated filters (3). This can become a significant problem when filtration is necessary for the preparation of sterile culture media that cannot be sterilised by heat due to sensitive media components or large volumes. Primary applications for mycoplasma filtration include large-scale mammalian cell culture media and bacterial cell culture broth used for aseptic process validation by sterile media fill. Common culture media components, such as serum and soy protein digests, may also become a source of mycoplasma contamination along with operator carriers (4).

Mycoplasma filtration generally requires the use of 0.1µm rated filters to provide a higher degree of removal efficiency than that afforded by 0.2 or 0.22µm rated filters. However, there is no industry standard for the rating of 0.1µm filters by filter manufacturers and not all 0.1µm rated filters are created equal. Test conditions applied by filter manufacturers to develop mycoplasma retention claims or examples may not be representative of the final user's product or process conditions, and therefore may not be as predictive of the efficiency of the process removal efficacy as presumed. Bacterial retentive filters (sterilising grade, mycoplasma retentive or otherwise) are not rated based on measurement of the pore size, but rather on the ability to retain an appropriate model organism (for example, a standard bacteria strain) under test conditions established by the filter manufacturer (for example modification of ASTM F835-05) (5).

TEST REQUIREMENTS

Given that bacterial retention testing is destructive (the filter cannot be used after testing), a non-destructive integrity test, such as a forward (diffusive) flow or bubble point test, is often developed and correlated to bacterial retention to provide a means of predicting or confirming retention. For a production filter cartridge or capsule to be described as a 'sterilising grade filter', it must have been tested prior to release by the filter manufacturer, using a test method correlated with a high degree of assurance to predict 100 per cent bacterial retention of the test bacteria at a challenge level of 1 x 10^7 colony forming units test bacteria/cm^2 of filter area.

Despite these tests, there are still concerns about potential bacterial penetration of an integral process filter during filtration of a drug product, which is why sterilising filters must also be tested for bacterial retention under drug product- and process-specific conditions to meet FDA requirements for sterile drug product good manufacturing practice (5,8). In process-specific filter validation, the filter medium is tested for bacterial retention using the actual drug product (or simulant) under 'worst case' conditions of process pressure and/or flow rate, temperature and any other relevant conditions. Worst case conditions are considered to be those most likely to facilitate bacterial penetration if any flow paths (or 'pores') through the membrane are large enough.

In contrast to sterilisation of the final drug product, however, mycoplasma filtration processes are often performed further 'upstream' (for example filtration of mammalian cell culture media or associated with aseptic process validation 'media fill'). These filtration processes are not specified in regulatory documents as requiring process-specific mycoplasma retention validation. In addition, as stated previously, there is no standard rating method for 0.1µm rated filters (the rating given to most filters with a mycoplasma retention claim); therefore test conditions and associated removal efficiencies may not be comparable among filter manufacturers, or accurately predictive of their performance in specific processes. In the end, users are left with little or no guidance on how to quantify and optimise mycoplasma clearance for their processes through the selection of filters 'nominally' rated at 0.1µm which the manufacturer has claimed to be mycoplasma retentive (to some degree).

In an attempt to address that gap, we present two important variables that a user should understand in making a filter selection in order to ensure mycoplasma clearance. The first is how the filter manufacturer measures and expresses the mycoplasma clearance for the filter (for example, the specified retention efficiency). The other is how this efficiency relates to performance in the specific user's process.

FILTER RATING & EFFICIENCY

Filters marketed with claims for mycoplasma retention are generally described as having a 0.1µm rating and may or may not have an assigned removal efficiency value (titre reduction or log reduction value) based on performance testing with a specified mycoplasma test organism – typically a strain of *Acholeplasma laidlawii* – under defined test conditions. The 0.1µm rating itself may be based on this mycoplasma retention efficiency, or may be otherwise based on the minimum bubble point value (relative to the filter manufacturer's 0.2µm membrane bubble point value), on retention of 0.1µm test particles, for example latex beads, under specified or unspecified test conditions, or on some other parameter. Mycoplasma filters are generally challenged (at a minimum) with *A laidlawii*, at a concentration of equal to or greater than 1 x 10^7 colony forming units (CFU)/cm^2 of filter surface area (comparable to ASTM-F838-05 recommendations for bacterial challenge of 0.2µm rated 'sterilising grade' filters), although some filter manufacturers may challenge with less. Some filters may also have been tested with other mycoplasma species (for example, *Mycoplasma orale*), as well as with the bacteria *Brevundimonas diminuta*, which is necessary for a claim of 'sterilising grade' according to regulatory GMP.

Although many filters cannot claim 100 per cent clearance of the challenge mycoplasma (no penetration at the specified challenge level and test conditions), this does not mean that they do not effectively retain a very significant amount of mycoplasma or are not highly efficient. It also does not necessarily mean that they will not be 100 per cent retentive in a given filtration process when considering the actual bacterial or mycoplasma bioburden load. To understand how that can be true, it is important to understand the concept of bacterial titre reduction (TR) (or log reduction value). TR is a measure of the degree to which a particular filter removes a microorganism under specified test conditions. TR is calculated as the ratio of the total number of bacteria used to challenge the filter divided by the total number of bacteria that passed through the filter:

$$TR = \frac{\text{Upstream titre x volume applied = total number of influent}}{\text{Downstream titre x volume filtered = total number of effluent}}$$

The TR expressed as a base 10 logarithm becomes the log reduction value (LRV).

In practical terms, this means that for any real likelihood of penetration to occur under process conditions with similarly penetrative bioburden, the bioburden load on the filter would have to exceed a total challenge of 100,000 to 1,000,000 CFU/cm^2 of filter area. However, an important caveat to keep in mind is that the TR is a product of the testing conditions and should be verified under specific process conditions. The higher the TR value demonstrated for a given filter, the greater the assurance of mycoplasma removal in the event of high mycoplasma bioburden levels (or more penetrative mycoplasma).

BACTERIAL CHALLENGE TESTING

In mycoplasma and bacterial challenge testing, the test organisms are preferably inoculated directly into the process fluid and delivered to the test filter(s) (0.2 or 0.1µm rated) and a control filter (the next 'coarser' grade) for a particular length of time, volume throughput, flow rate and/or pressure, and temperature. The test and control filter(s) are subjected to pre- and post-challenge integrity or installation tests, generally a Forward (diffusive) Flow test or a bubble point test. When 47mm discs are used (as is often the case), integrity testing generally includes a bubble point test to demonstrate the correct installation of the filter into the housing, and ensure there are no gross defects with each filter. With larger filter modules (such as 10 inch cartridges or capsules), the integrity testing is more typically conducted using Forward (diffusive) Flow testing. This is especially true for 0.1µm rated filters that may have water wet bubble point values more than 90psi (six bar).

The test bacteria should be suspended in the product at a concentration and in a volume that delivers a challenge level that meets or exceeds the industry minimum standard (for 0.2µm rated sterilising grade filters) of 1 x 10^7 CFU/cm^2 of test filter area. The effluent concentration (or total recovery) is also determined.

There are two possible outcomes to a bacterial challenge: either no penetration occurs and the filter is considered 'sterilising' (under those testing conditions it is 100 per cent retentive); or there is some degree of penetration (also under the given testing conditions). If there is some degree of penetration, then the TR or LRV is calculated as described above. When no penetration occurs, the TR or LRV may be expressed as greater than the total challenge level for the entire filter area.

PRODUCT-SPECIFIC (USER) FILTER VALIDATION

When bacterial challenges are conducted by the filter manufacturer the challenge is generally administered in a single pass with a simple buffer or water, commonly at a relatively low pressure and/or flow rate in a convenient volume so as to meet the predetermined challenge level, as described in ASTM Standard F838-05 (5). As mentioned previously, a filter user's product or process conditions may be different from that of the filter manufacturer's core validation conditions, and may influence performance. This is why process-specific filter validation is important for sterilising filtration. Since regulations do not implicitly require process-specific mycoplasma filtration validation, when evaluating mycoplasma filters, it is prudent to consider how the filter was tested by the filter manufacturer to establish the reported rating and the reported TR (especially if no product-specific filtration validation will be conducted).

PROCESS-SPECIFIC MYCOPLASMA CHALLENGE TEST

In contrast to the manufacturer's core retention validation study, the aim of a process-specific study is to generate data demonstrating that the product filtration process will remove mycoplasma effectively when the product is passed through a mycoplasma filter under the actual processing conditions. It will determine the mycoplasma clearance efficiency under the user's actual process conditions.

Process-specific mycoplasma retention validation is accomplished by inoculating mycoplasma directly into the product (or where necessary, an appropriate simulant), and then passing the spiked product through the test filters under conditions designed to simulate the worst-case process parameters of a full-scale production run. Prior to performing the process-specific mycoplasma challenge test, it may be necessary to determine the viability of the challenge test organism in the process fluid.

The mycoplasma challenge should provide a uniform challenge over the intended process time to yield a final challenge level of at least 10^7 CFU/cm^2 of filter surface area (6). Retention testing will suffer sensitivity loss directly proportional to the degree of cellular aggregation, so the latter must be assessed and controlled. Absence of significant aggregation and appropriate mycoplasma cell sizing for qualification of a 0.1µm-rated filter is generally confirmed by demonstrating penetration of a 0.2µm-rated membrane as a positive control for each challenge test performed.

The test conditions should simulate the production process and, as much as possible, represent worst-case testing conditions. If the filtration process is regulated by pressure, the challenge pressure used during testing should be equal to the maximum processing pressure. Volume throughputs should be scaled appropriately to meet or exceed the maximum batch size processed in the full-scale operation. Also, the duration of the challenge should meet or exceed the maximum amount of time that the filter comes into contact with the process fluid (if possible).

Three different filter membrane lots are generally included in the product-specific microbial retention validation study. It is important to include at least one filter lot representative of filter material with the largest acceptable pore size in order to demonstrate that microbial retention is adequate by any membrane in the filter grade. This 'worst case' membrane should have a physical integrity test value at or near the manufacturing specification for that filter representative of the largest 'pores'. Typically, this 'worst case' filter will have a manufacturer's 'bubble point' within 10 per cent above the minimum filter manufacturing specification. The physical installation of the challenge membrane in the disc holder is also determined prior to challenge testing by a visual bubble point test, using water or other wetting fluid for which specifications exist.

FACTORS INFLUENCING MYCOPLASMA RETENTION

As evident in the discussion above, it is assumed that flow rate, pressure, temperature and other process parameters or product formulation characteristics may influence bacterial or mycoplasma retention. They are reasonable assumptions, however, exactly how, or if, these factors influence mycoplasma retention/penetration of filters has, for the most part, not been publicly documented. On the other hand, there is strong evidence that some characteristics of product solutions do play a significant role in bacterial penetration of sterilising grade filters. For example, solutions with a high lipid content and a low surface tension (such as a liposome solution) have been shown to enhance filter penetration by *B diminuta* under conditions that would not otherwise normally permit penetration (9). It would therefore not be surprising if mycoplasma penetration is also enhanced in similar solutions.

One factor that has clearly been shown to influence bacterial penetration of filters is the growth media used to generate the challenge test organism. This effect is most likely due to the fact that bacteria will adapt to environmental conditions in an attempt to enhance their survival and these adaptations may include a change in cell size or other cell characteristic. A wide variety of bacteria are typically reduced in size subsequent to starvation (10-13). Unsurprisingly, this is also true of mycoplasma cells, for example, the diameter of mycoplasma cells grown in the presence of glucose has been reported to be two to four times larger than cells grown in the absence of glucose (1 to 3µm compared to 0.5 to 1µm) (14).

Given that the mycoplasma cell nutritional status may also affect the penetrative ability of the challenge test mycoplasma, the effect of culture media on mycoplasma penetrative ability has been studied (to a limited extent). These preliminary studies have shown that mycoplasma culture conditions can impact the apparent retention efficacy of challenged filters. For example *A laidlawii* mycoplasma cells cultured in mycoplasma broth with 10 per cent horse serum were demonstrated to penetrate a 0.2µm rated filter more effectively than those cells cultured in tryptic soy broth alone (3). This is notable given that many of the fluids filtered using mycoplasma filters are well suited to mycoplasma growth.

CONCLUSION

Similar to the challenge study used for validating sterilising grade filters (0.2 or 0.22µm rated) for 100 per cent bacterial removal using *B diminuta* multiple factors are considered when designing a process-specific mycoplasma retention

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validation study, and should be compliant with the sterilising filter validation methods described by ASTM International and the Parenteral Drug Association (5,6,15). The factors to be considered include:

- Filter type (structure, base polymer, surface modification chemistry, pore size distribution and thickness)
- Fluid components (formulation, surfactants and additives)
- Fluid properties (pH, viscosity, osmolality and ionic strength)
- Process conditions (temperature, pressure differential, flow rate and time)
- The specific characteristics of the actual bioburden in the product (if applicable)

By using the actual process fluid and simulating the full-scale processing conditions, these factors are accounted for during the testing.

Given that how a filter is challenge tested for mycoplasma retention can influence the outcome of the testing (0.1µm rating, claimed mycoplasma TR or LRV), it is important to consider how a filter was tested when comparing ratings and titre reductions reported by filter manufacturers. Ultimately, for increased assurance that a filter will perform as needed, process-specific mycoplasma retention validation is recommended.

References

1. Drexler HG and Uphoff CC, Mycoplasma contamination of cell cultures: Incidence, sources, effects, detection, elimination and prevention, *Cytotechnology* 39(2): pp75-90, 2006
2. Potts B, Proceedings from the PDA Workshop on Mycoplasma Contamination by Plant Peptones, *PDA: San Diego, California*: pp1-257, 2007
3. Folmsbee M, Howard G and McAlister M, Nutritional effects of culture media on mycoplasma cell size and removal by filtration, *Biologicals* 38(2): pp214-217, 2010
4. Windsor HM, Windsor GD and Noordergraaf JH, The growth and long term survival of *Acholeplasma laidlawii* in media products used in biopharmaceutical manufacturing, *Biologicals* 38(2): pp204-210, 2010
5. ASTM, Standard Test Method for Determining Bacterial Retention of Membrane Filters Utilized for Liquid Filtration American Society for Testing and Materials (ASTM), ASTM Standard F838-05, 2005
6. PDA, PDA Technical Report No 26, Sterilizing Filtration of Liquids, *PDA J Pharm Sci Technol* (S-5): pp1-60, 2008
7. FDA, Guidance for Industry: Sterile Drug Products Produced by Aseptic Processing – Current Good Manufacturing Practice. In Food and Drug Administration (FDA), FDA: Rockville, MD, 2004
8. Cooney P, FDA, CDER, OPS Microbiology in PDA/FDA Forum: 1995
9. Onraedt A, Folmsbee M, Kumar A and Martin J, Sterilizing filtration of adjuvanted vaccines: ensuring successful filter qualification, *BioPharm International*, October 2010
10. Velimirov B, Nanobacteria, ultramicrobacteria and starvation forms: a search for the smallest metabolizing bacterium, *Microbes and Environment* 16(2): pp67-77, 2001
11. Sargent M, Control of cell length in *Bacillus subtilus*, *J Bacteriol* 123: pp7-19, 1975
12. Donachie WD and Begg KJ, Cell length, nucleoide separation and cell division of rod-shaped and sherical cells of *Echerichia coli, J Bacteriol* 171: pp4,633-4,639, 1989
13. Schaechter M, Maaloe O and Kjeldgaard NO, Dependency on medium and temperature of cell size and chemical composition during balanced grown of *Salmonella typhimurium*, *J Gen Microbiol* 19(3): pp592-606, 1958
14. Anderson DL, Pollock ME and Brower LF, Morphology of Mycoplasma *laidlawii* Type A II, Effect of glucose on growth and cellular morphology, *J Bacteriol* 90(6): pp1,768-1,777, 1965
15. FDA, Sterile Drug Products Produced by Aseptic Processing, In FDA Guidance for Industry: 2004

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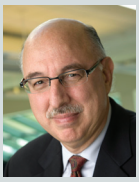

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