

Towards an Improved Sterility Test

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The sterility test is generally recognized as a flawed test for its stated purpose. Since the original description in 1932, this test has generated controversy as to its role in product quality testing and in terms of means to improve the assay. As early as 1956 Bryce published an article describing the two critical limitations of this test (1). He put forward that the test was limited in that it can only recognize organisms able to grow under the conditions of the test, and that the sample size is so restricted that it provides only a gross estimate of the state of "sterility" of the product lot. Other concerns about the sterility test (e.g., choice of sample size, choice of media, time and temperature of incubation) were extensively reviewed in an article by Bowman (2).

There have been several changes in the compendial sterility test since that time, culminating in the internationally harmonized test (3). However, the two basic problems outlined in 1956 by Bryce remain today.

Sample Size

The sample size is set arbitrarily, and does not provide a statistically significant population to estimate sterility (4). This is indisputable and unavoidable with a test of this type, which is destructive in nature. Let's look at some of the numbers:

Let the likelihood of a contaminated unit = λ

By the Poisson distribution, the probability of picking a sterile unit from the fill (denoted P) is $e^{-\lambda}$, or 2.7182818^{- λ}

Then, if you are picking 20 samples from an infinite supply (or, for this discussion, from a pharmaceutical batch):

The probability of passing the sterility test is P^{20}

Conversely, the probability of failing the Sterility Test is $1 - P^{20}$

So, if we were to calculate the likelihood of the sterility test actually failing, given a known frequency of contaminated units in the batch:

Frequency of Contaminated Units in the Batch	Probability of Failing Sterility Test with the Current Sample Size
0.001	0.0198–2%
0.005	0.0952–9.5%
0.01	0.1813–18%
0.05	0.6321–63.2%
0.1	0.8647–86.5%
0.5	1.0000–100%

The only way to improve the likelihood of finding a failure is by significantly modifying the sampling plan. This change is extremely unlikely for several reasons. A discussion of different sampling plans that might be used is presented in Bryce (1), and a more full discussion of the controversy over the final resolution of the current procedure is provided in Bowman (2). After extensive review, all of the proposed sampling plans were found wanting for reason or another. Changing the sample plan, and the sample size, have proven to be an unproductive avenue for change.

One frequently overlooked aspect of discussions of sampling plans is that the statistical analyses all assume that the test system would recover even a single microorganism if it were present in the sample. In other words, one contaminating cell would result in media turbidity. This assumption leads us to the next topic.

Recovery Conditions

The harmonized test utilizes Typticase Soy Casein Digest Broth and Fluid Thioglycollate Medium.

These media and their corresponding incubation temperatures were chosen to maximize recovery of potential contaminants early in the development of the tests. However, some authors have questioned the choice of media (5), while others have suggested that the use of solid media rather than liquid media would be appropriate (6). The choices in the current harmonized procedure reflect those media to which all parties in the harmonization process could agree.

Then there is the concern about incubation duration. The *United States Pharmacopeia*, 23rd edition (1995) (7) allowed a 7-day incubation period for products tested by membrane filtration, 14 days for those tested by the direct transfer method. This requirement changed in *United States Pharmacopeia*, 24th edition (2000) (8) to include a 14-day incubation period for both types of tests. Similarly, the *European Pharmacopoeia*, 3rd ed. (1997) (9) allowed a 7-day incubation period (unless mandated by local authorities), but in 1998 the 4th edition had changed this to 14-days for both. This extension was the result of concerns that the methodology might not be able to detect "slow-growing" microorganisms.

The incubation period was identified as a concern by Ernst et al. (10), who recommended a longer period of incubation time than 7 days might be necessary, perhaps as long as 30 days. More recently this position was repeated with retrospective data provided by German and Australian workers who wished to ensure that a harmonized procedure included an incubation period of at least 14 days (11, 12).

However, even with the longer incubation period there is no assurance that all microorganisms can grow under these conditions, but are metabolically active. In fact a growing body of evidence suggests that there are a large number of microorganisms that are unable to replicate under standard laboratory conditions (Viable But Not Culturable – VBNC) (13–15).

Possible Improvements to the Sterility Tests

So where does that leave the industry in the effort to improve the sterility test methodology? The sample size question does not admit to an easy answer after 70 years of evaluation. Despite the obvious issues with the sample size, this characteristic of the sterility test does not seem amenable to change, especially now that the compendial test has been harmonized across

all the pharmacopeia (3). That leaves a single avenue to explore, that of increasing the sensitivity of the recovery conditions.

One way to improve recovery conditions would be to evaluate different forms of media (e.g., solid versus liquid) or changing the media compositions in order to increase their potential in improving microbial recovery. There is a substantial database of research that has been performed regarding this topic and it is unlikely to yield dramatic improvements over the existing methods.

Another way to improve recovery conditions would be to change the rules. Rather than require growth of the contaminant sufficient to turn media turbid, perhaps a different indicator of microbial viability could be used in the sterility test. This technology is available within the realm of rapid microbiological methods.

Rapid Microbiological Methods of Potential Use

Currently marketed rapid microbiology methods can be grouped into two types—those that require amplification (growth) to show low-level contamination, and those that do not. In the first group would be technologies such as adenosine triphosphate (ATP) bioluminescence, head-space analysis, and others. Examples of the second type might be technologies such as polymerase chain reaction (PCR) and vital dye/chromatography methods. Why is this distinction important?

The concern with recovery conditions is that we do not know how to grow all microorganisms that might contaminate pharmaceutical products. Applying an alternate technology that requires growth does not result in an improvement in the sterility test method, since organisms that currently do not grow would not grow in the new method either (assuming the use of similar growth conditions in the new test). In addition, there is the continuing concern about the duration of the incubation period.

The currently required 14-day incubation period imposes a significant burden on the manufacturer, who must quarantine product until successful completion of the test. Can this be shortened in an alternate test? The time required for microbial growth to turbidity can be thought of as the sum of two stages, a lag phase where the microorganism prepares to grow, and the generation time requirements for a low level of microorganisms to grow to a concentration where they are visible using human vision, that is, approximately

10⁷ cfu/mL. This separation of stages is important, as it seems that the lag phase is the most significant portion of time required for turbidity (16). Therefore any alternate methodology that requires growth to amplify the microorganism should be assumed, unless proven otherwise, to require a lengthy incubation period to ensure the recovery of "slow-growing" microorganisms. This limitation can be avoided by use of a technique that does not require growth.

The use of a method that avoids growth requirements offers an additional advantage in that the question of VBNC organisms is completely side-stepped. As no culturing is required, the recovery phase of the sterility tests can be optimized to all microorganisms regardless of growth requirements.

Conclusions

The current, harmonized sterility test has two fundamental weaknesses, both of which have been obvious from its inception. The first is that the sampling plan is insufficient to meet the requirements implied by the title of the test. This weakness is not solvable in the current regulatory climate (nor has it been for over 70 years). The second weakness of the test involves recovery and recognition of microbial contamination in the sample, should it exist. Using growth-based technologies, the sterility test incubation period has been expanded to 14 days. Currently available alternate technologies that do not require growth offer the opportunity to dramatically improve the sensitivity and ease of use of the test. They also offer the pharmaceutical manufacturer the potential to reduce product quarantine period from weeks to days.

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