QUALITY CONTROL ANALYTICAL METHODS:

Microbial Limit Tests for Nonsterile Pharmaceuticals, Part 1

Nicole Vu, PhD
Jessica R. Lou, BS
Thomas C. Kupiec, PhD

This article represents part 1 of a 2-part article on the topic of microbial limit tests for nonsterile pharmaceuticals. Part 1 provides valuable information on this topic, including an overview of United States Pharmacopeia Chapter <61>. Part 2 continues with this discussion, including an overview of United States Pharmacopeia Chapter <62>. Both parts of this series of articles contain information integral to contamination control.

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ABSTRACT **Contamination of pharmaceuticals with microorganisms may lead to deleterious effects on the therapeutic properties of the drug, and may potentially cause injuries to intended recipients. Cases of contaminated nonsterile products have been reported in increasing numbers, and often associated with the presence of objectionable microorganisms. Methods for detection of these organisms are described in three major Pharmacopeias. Their functions and their limitations in the examination of microbiological quality for nonsterile products will be reviewed in this report.**

Nicole Vu *and* ***Thomas C. Kupiec*** *are affiliated with Analytical Research Laboratories, Inc., Oklahoma City, Oklahoma;* ***Jessica R. Lou*** *is a PharmD Candidate at the Oklahoma University Health Science Center, Oklahoma City, Oklahoma.*

Nonsterile pharmaceuticals are not produced by aseptic processes and, therefore, are not expected to be totally free from microbial contaminations. The degree of contamination in nonsterile products is regulated, and is based on the acceptance criteria for microbiological quality established in Pharmacopeial monographs. A review of the U.S. Food and Drug Administration's (FDA) enforcement reports during 2004–2011 revealed that approximately 75% of nonsterile product recalls were in fact due to contaminated over-the-counter (OTC) or personal care products. The majority of these recalls were attributed to the following[1]:

- Presence of "objectionable" organisms (72%)
- Contamination levels exceeding microbial limits (15%)
- Sterility or microbial diagnostic kit errors (7%)
- Failed microbiological tests (5%)
- Manufacturing deficiencies (1%)

The FDA has indicated that "topical preparations contaminated with Gram-negative organisms are a probable moderate to serious health hazard," and that "*Burkholderia (Pseudomonas) cepacia* is objectionable if found in a topical product or nasal solution in high numbers." The FDA concerns were obviously related to past incidents, where various infections and deaths were linked to contaminated Povidone-Iodine products, and Metaproterenol Sulfate Inhalation Solution.[2] Since then, aqueous-based inhalants are required to be sterile.[1]

The major contaminants of nonsterile pharmaceutical products and ingredients are bacteria, yeast, and molds. Even dry formulations are susceptible to microbial contamination as the proliferation of microorganisms in solid dosage forms have been observed, especially in warm and humid climates. There are very few

products that possess self-preserving characteristics such as syrups, elixirs, and spirits; other multi-use pharmaceuticals usually contain antimicrobial preservatives to improve product safety.[3]

Non-preserved drug products used in a hospital pharmacy for dose preparation have their own limitations even when they are supposed to be sterile. The final preparations are held for an extended period of time before administration to the patient, and are susceptible to microbial growth during the holding period.[4] Poor aseptic techniques have been linked to nosocomial outbreaks including *S. aureus*- and *Enterobacter*-laden propofol[5] and *Enterobacter*- and *P. aeruginosa*-contaminated dextrose vials for multi-dose use.[6] The propofol and dextrose tragedies each contributed to two deaths. Propofol, an intravenously-administered anesthetic, is available in both a preservative (metabisulfite or disodium edetate) and non-preservative formulation. The low-lipid emulsion formulation is not preserved and promotes rapid microbial growth at room temperature.[7] Similarly, any product that is available in a dosage form intended for use in more than one patient (multi-dose) is worrisome for encouraging microbial growth. While such dosage forms afford distinct cost-saving and convenience advantages, contamination in these "multi-dose" containers pose a significant risk as a source for nosocomial infections.[8]

Under current Good Manufacturing Practices (cGMP), manufacturers are expected to maintain strict adherence to microbial contamination control practices during the production, and to develop microbial specifications for their nonsterile products.[9] The challenge for manufacturers, a challenge that may include outsourcing facilities, is to produce a nonsterile dosage form that does not exceed the recommended limit for microbial load, and is not contaminated with objectionable microorganisms.[1]

Objectionable microorganisms may be pathogens or opportunistic pathogens with their attendant metabolic activities and their microbial characteristics such as exotoxins, endotoxins, sporulation, etc. These microorganisms can grow under sub-optimal temperature and nutrients and may affect product quality and safety. Contamination at any steps in the manufacturing or compounding process represents significant risks to that process, which must be controlled to protect product quality and safety.[10]

United States Pharmacopeia (USP) Chapters <61> *Microbiological Examination of Non-Sterile products: Microbial Enumeration Tests* and <62> *Microbiological Examination of Non-Sterile products: Tests for Specified Microorganisms* provide protocols that allow quantitative enumeration of the presence of bacteria and fungi. The tests help determine whether a nonsterile product complies with an established specification for microbiological quality. Additionally, these two *USP* chapters provide guidance on determining the absence of, or the limited occurrence of, specified microorganisms that may be detected under the conditions of the tests.[11] It is necessary to emphasize here that the *USP* provides methodologies for selected indicator organisms, but not all "objectionable" organisms in the FDA opinions.[2]

ACCEPTANCE CRITERIA FOR MICROBIOLOGICAL QUALITY OF NON-STERILE PHARMACEUTICALS

The microbial limits recommended in *USP* General Chapter <1111> *Microbiological Examination of Non-Sterile products: Acceptance criteria For Pharmaceutical Preparations and Substances For Pharmaceutical Use* are based on the total aerobic microbial count (TAMC), total combined yeasts and molds count (TYMC), and tests for absence of the specified organisms by route of administration (Table 1).[11] The *USP* expresses the following conditions in applying the acceptance

criteria for evaluation of product quality:

1. The microbiological test methods are highly variable and must be validated with a limit of detection as close as possible to the indicated acceptance criteria.
2. The list of microorganisms in Table 1 is not exhaustive: The significance of other microorganisms recovered should be evaluated in terms of route of administration, the nature of the product (e.g., growth promotion properties), the method of application, the intended recipient (neonates, infants, debilitated conditions, etc.), the use of immunosuppressive agents, and the presence of disease or organ damage.
3. Acceptance criteria are applied to individual results or the average of replicate counts in colony-forming units per gram or mL of the product (cfu/g or cfu/mL). The maximum acceptable range for microbial enumeration is 2 times (or ± 0.3 $\log_{10}$) the limit. For example, results for a TAMC ranging from 5–20 cfu/mL would meet the specification of 10 cfu/mL.

OVERVIEW OF *USP* CHAPTER <61>: MICROBIAL ENUMERATION TESTS

USP Chapter <61> provides tests for the quantitative determination of total aerobic microbial count, and TYMC that might be present in pharmaceutical ingredients and finished products.[11] These methods are not applicable to products containing viable microorganisms as active ingredients. Alternate procedures may be used, but must show to be equivalent to Pharmacopeial methods. All aspects of the test are conducted under conditions designed to limit extrinsic contaminants from personnel, environment, reagents, or glassware.

Antimicrobial activities inherent in the test sample must be removed or neutralized, and the applied method must be non-inhibitory to microbial growth through demonstration of adequate recovery for representative

TABLE 1. *United States Pharmacopeia* (Chapter <1111>) Acceptance Criteria for Microbiological Quality of Nonsterile Dosage Forms.[11]

ROUTE OF ADMINISTRATION	TAMC (CFU/G, CFU/ML)	TYMC (CFU/G, CFU/ML)	ABSENCE OF SPECIFIED MICROORGANISM(S) (1 G, 1 ML)[a]
Oral (non-aqueous)	10^3	10^2	*Escherichia coli*
Oral (aqueous)	10^2	10^1	*Escherichia coli*
Rectal	10^3	10^2	None designated
Oromucosal	10^2	10^1	*Staphylococcus aureus* *Pseudomonas aeruginosa*
Gingival	10^2	10^1	*Staphylococcus aureus* *Pseudomonas aeruginosa*
Cutaneous	10^2	10^1	*Staphylococcus aureus* *Pseudomonas aeruginosa*
Nasal	10^2	10^1	*Staphylococcus aureus* *Pseudomonas aeruginosa*
Auricular	10^2	10^1	*Staphylococcus aureus* *Pseudomonas aeruginosa*
Vaginal	10^2	10^1	*Pseudomonas aeruginosa* *Staphylococcus aureus* *Candida albicans*
Transdermal Patch (drug matrix, adhesive layer and backing)	10^2	10^1	*Staphylococcus aureus* *Pseudomonas aeruginosa*
Inhalation	10^2	10^1	*Staphylococcus aureus* *Pseudomonas aeruginosa* Bile-tolerant Gram-negative bacteria
Pharmaceutical substances	10^3	10^2	None designated

[a]Minimum amount of product to be used in sample preparation; cfu = colony-forming unit; TAMC = total aerobic microbial count; TYMC = total combined yeasts and molds count

microorganisms in validation testing. Microbial recovery is enumerated by one of three prescribed methods: 1) Membrane Filtration, 2) Plate Count (PCM) (pour-plate or spread-plate techniques), or 3) Most Probable Number (MPN). The MPN method is reserved for TAMC in low bioburden samples, and is not suitable for the estimation of fungal recovery.

USP CHAPTER <61> METHOD SUITABILITY TEST (METHOD VALIDATION)

The suitability test is conducted to demonstrate the applicability of the method for detection of microbial contamination in the test product. Validation testing is usually performed prior to product testing using a panel of five representative microorganisms as indicators. Concurrent validation and product testing are possible based on the product history, and must be performed prior to product release. The compositions of required diluents and media are described in *USP* Chapter <62>.

Test Organisms and Preparation of Standardized Cell Suspensions

A panel of five representative microorganisms is used in the validation of *USP* <61>, including:

- *Staphylococcus aureus* (Gram-positive coccus)
- *Pseudomonas aeruginosa* (non-fermentative Gram-negative bacillus)
- *Bacillus subtilis* (spore-forming Gram-positive bacillus)
- *Candida albicans* (yeast)
- *Aspergillus (niger) brasiliensis* (mold)

A list of the approved strains and their commercial sources is provided in Table 2. Fresh cultures of each organism are harvested and standardized to an optical density of 0.1–0.3 at 550 nm in Buffered Sodium Chloride-Peptone Solution (pH 7.0), or Phosphate Buffer Solution (pH 7.2). Seed-lot systems are recommended for long-term storage, and stock culture of each organism is limited to no more than five passages removed from the master seed-stock. The standardized cell suspensions should be used within 2 hours or they must be stored at 2°C to 8°C for not more than 24 hours. Stable spore suspensions of *A. brasiliensis* or *B. subtilis* may be substituted for vegetative cell suspensions and maintained at 2°C to 8°C for a validated storage time.

Microbial enumeration test is performed to determine the number of viable cells in each cell suspension. In general, bacteria are grown at 30°C to 35°C on Soybean-Casein Digest (SCD) Agar, while yeast and mold are grown at 20°C to 25°C on Sabouraud Dextrose Agar (SDA) or Potato Dextrose Agar (PDA). The culture conditions in Table 3 are for preparation of standardized cell suspensions and for microbial recovery in the validation studies.

Preparation of Test Sample

The amount of sample to be examined by *USP* <61> is generally 10 g or 10 mL of the product. Otherwise, composite samples of randomly selected dosage units are examined under the following conditions:

- Aerosol dosage forms: 10 containers
- Transdermal patches: 10 patches
- Tablets, capsules, injections: 10 units
- Drug substances: 1% of the batch if less than 1000 mL or 1000 g
- Clinical trial samples: 1% or 1 to 2 units for batch sizes between 100 to 200 units

In all cases, the sampling plan should reflect the status of the product. A biased sampling plan cannot be used because contaminants are not distributed uniformly in the batch. The test sample should be a mixture of at least three equal portions taken at random to represent the production batch as much as possible.[12]

TABLE 2. Representative Microorganisms for Use in Validation of *United States Pharmacopeia* Chapters <61> and <62>.[11]

ORGANISM	ATCC	NCIMB	CIP	NBRC	NCTC	NCPF	IP
Staphylococcus aureus	6538	9518	4.83	13276	NA	NA	NA
Pseudomonas aeruginosa	9027	8626	82.118	13275	NA	NA	NA
Bacillus subtilis	6633	8054	52.62	3134	NA	NA	NA
Candida albicans	10231	NA	NA	1594	NA	3179	48.72
Escherichia coli	8739	8545	53.126	3972	NA	NA	NA
Salmonella enterica subsp: *serovar* typhimurium or *serovar* abony	14028 NA	NA	NA 80.39	NA 100797	NA 6017	NA	NA
Clostridium sporogenes	11437 or 19404	12343	100651 or 79.3	14293	532	NA	NA

NA = not available

USP Chapter <61> provides guidelines for the preparation of test sample depending on the dosage forms and their formulation characteristics (Table 4). Alternative sampling methods for inhaled and nasal products are described in *USP* Chapter <610>.[11] In microbial enumeration test, a homogeneous solution or suspension of the product is prepared for the test at 1:10 dilution ratio in a suitable sterile diluent. The dilution ratio may be adjusted to accommodate poorly soluble products or due to the presence of inhibitors, but only if method sensitivity and acceptable recovery is demonstrated for each representative microorganism. Diluents commonly used in microbiological tests are Buffered Sodium Chloride-Peptone Solution (pH 7), Phosphate Buffer Solution (pH 7.2), or SCD or TSB. A surface-active agent may be used to enhance solubility, suspendability, or emulsification of water insoluble products. As previously discussed, any antimicrobial properties of the drug product must be neutralized to recover viable cells in the microbial enumeration test. This neutralization may be accomplished by neutralizing agents, membrane filtration, dilution, or any combination of these methods. If the product is inherently microbicidal towards the test strains and neutralization is not

TABLE 3. Culture Conditions for Preparation of Standardized Cell Suspensions and for Microbial Recovery Studies in Method Validation Experiment (*United States Pharmacopeia* Chapter <61>).[11]

ORGANISM	CULTURE MEDIUM	TEMP (°C)	TIME (CELL SUSPENSION)	TIME (RECOVERY)
S. aureus	Soybean-Casein Digest (broth, agar)	30 to 35	18 to 24 hours	≤3 days
P. aeruginosa	Soybean-Casein Digest (broth, agar)	30 to 35	18 to 24 hours	≤3 days
B. sutillis	Soybean-Casein Digest (broth, agar)	30 to 35	18 to 24 hours	≤3 days
C. albicans	Sabouraud Dextrose (agar, broth)	20 to 25	2 to 3 days	≤5 days[a]
A. brasiliensis	Sabouraud Dextrose Agar or Potato-Agar	20 to 25	5 to 7 days	≤5 days[a]

[a]Alternate medium: Sabouraud Dextrose Agar or Soybean-Casein Digest Agar

achievable, then the enumeration method should be conducted at the highest dilution factor compatible with microbial growth to detect other microorganisms that might be present in the product. Table 5 includes examples of commonly utilized neutralizing agents which may be added to the diluent or the medium prior to sterilization.[3,11]

Validation of Microbial Recovery in the Presence of Product

The validation study must show that recovery of an inoculum containing ≤100 cfu of the representative organism is not inhibited by the test sample and by the neutralization method. This is accomplished by comparing recovery results for three treatment groups:

1. The test group: Neutralized product inoculated with 100 cfu of the challenge organism
2. The peptone control group: The same treatment as in the test group but peptone is used instead of the test product
3. Inoculum control: Containing 100 cfu of the challenge organism, but no neutralization and no product present

The validation study is conducted in three independent experiments as described in *USP* <61>. The volume of inoculum added to each test samples should be within 1% of the volume of diluted product. For TAMC, the SCD agar plates are incubated at 30°C to 35°C for not more than three days, while the SDA plates for TYMC are incubated at 20°C to 25°C for not more than five days (Table 3). In the Membrane Filtration Method, a single SCD and SDA plate is prepared for TAMC and TYMC using a 10-mL aliquot of the diluted sample preparation for each medium. Whereas in the PCM, duplicate culture plates are used for each medium, and average cfus are reported for TAMC and TYMC. The volume of sample used in the Pour-Plate method is 1 mL, and 0.1 mL of sample preparation is used in the Spread-Plate method (Table 6). The Pour-Plate method is therefore more sensitive than the Spread-Plate method, and both PCMs are less sensitive than the Membrane Filtration method. When using these three methods, the TAMC and TYMC values for the test group and the control groups should not differ by a factor greater than 2 (or greater than ± 0.3 $\log_{10}$).

USP Chapter <61> suggests reserving the MPN method for situations where no other methods are available for the estimation of TAMC. The MPN method is not suitable for TYMC and is the least precise of all prescribed methods. The MPN method is conducted with a minimum of three serial 10-fold dilutions of the product using Tryptic Soy Broth (TSB or SCD broth) that represent 0.1, 0.01, and 0.001 g (or mL) of the product. At each dilution level triplicate tubes are prepared and incubated at 30°C to 35°C for not more than 3 days. Development of turbidity in any tubes is positively scored as indication for microbial growth. If the product is inherently turbid and interferes with visual evaluation, then sub-culture in the same medium and further incubate tubes for 1 to 2 days. The results are expressed as the most probable number of TAMC per g (or mL) of the product. When using the MPN method, the MPN value for the test

group must be within 95% confidence limits of the values obtained with the inoculum control. Chapter <61> includes a table of MPN values and their Confidence Limits for interpretation of results obtained by the MPN method. The negative control or reagent blank samples must exhibit no growth, irrespective of method used. A failed negative control requires an investigation.

Testing of Products by USP Chapter <61>

The TAMC and the TYMC are determined using the validated method. When testing the product by the Membrane Filtration Method, two culture plates are prepared, one SCD plate for TAMC and one SDA plate for TYMC; when testing product by the Plate Count Methods, duplicate SCD plates for TAMC and duplicate plates of SDA for TYMC are prepared. The SCD plates are incubated at 30°C to 35°C for 3 to 5 days, and the SDA plates are incubated at 20°C to 25°C for 5 to 7 days.

It should be observed that the incubation time for product testing is two days longer than the conditions used in the validation of method. This longer incubation time provides a better survival condition for damaged or slow-growing cells.[2] The general stipulations in *USP* <61> require that culture plates should be incubated to reach the highest growth level within the countable range, but they should be inspected early for overgrowth that might interfere with enumeration activities. The maximum countable range for TAMC is about 250 cfu, and about 50 cfu for TYMC on the standard 9-cm diameter petri plates.

TABLE 4. Recommended Sample Preparation for Microbiological Tests (*United States Pharmacopeia* Chapter <61>).[11]

PRODUCT	STERILE DILUENT
Water soluble	Buffered Sodium Chloride-Peptone Solution (pH7), or Phosphate Buffer Solution pH 7.2, or Soybean Casein Digest Broth
Non-fatty, water insoluble	Diluent containing 0.1% Polysorbate 80 or other surfactant
Fatty product	Dissolved in isopropyl myristate or other surfactant solution with homogenization and heat
Aerosols	Follow *United States Pharmacopeia* <610>
Transdermal patches	Remove the release liner and shake in diluent containing inactivators for 30 minutes

Interpretation of Results

The TAMC value for the product is based on the number of colonies found in the SCD medium, including any fungal colonies. Similarly, if bacteria colonies are found in SDA medium, they are counted as part of the TYMC for the product. Average plate counts are determined for the replicate plates and results are reported as the number of cfu per g (or per mL) of the product after adjusting for any dilution factor. The limit of detection is 1 cfu on solid media, and this limit of detection is multiplied by the dilution factor for reporting a zero count. For example, when plating a 1-mL aliquot of 1:10 diluted product in the Pour-Plate method, a zero result on the SCD plate should be reported as less than 10 cfu (<10 cfu) per g or mL of the product.

The TAMC and TYMC results are evaluated based on the acceptance criteria for the product group (Table 1). *USP* <61> specifies that the maximum acceptable count for the product is twice the expressed limit to account for method variability, such that a product with TAMC of 200 cfu per g would meet the microbial limit criteria 10^2 cfu per g.

SUMMARY

The microbial limit for nonsterile products must be within an acceptable range that does not pose health hazards to intended patient groups or diminish product stability. Objectionable organisms can be detected using procedures described in *USP* <61>, which is discussed in this article (part 1), and *USP* <62>, which will be discussed in part 2.

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TABLE 5. Recommended Neutralizing Agents.[3,11]

ANTIMICROBIAL AGENT	NEUTRALIZING METHOD
Alcohols	Dilution, Polysorbate
Aldehydes	Dilution, Thiosulfate, Glycine
Bis-biguanide	Lecithin
Chlorhexidine	Lecithin, Polysorbate
EDTA (edetate)	Mg^{2+} or Ca^{2+} ions
Glutaraldehyde	Glycine, Sodium bisulfite
Halogens	Thiosulfate
Iodine	Polysorbate
Mercuric chloride	Thioglycollate, Thiosulfate
Mercurials, other	Thioglycollate, Thiosulfate
Parahydroxybenzoates	Lecithin, Polysorbate
Phenolics	Dilution, Polysorbate and Lecithin
Quaternary Ammonium	Lecithin, Polysorbate
Sodium hypochlorite	Sodium thiosulfate
Sorbates	Dilution

TABLE 6. Summary of Validation Experiment by Membrane Filtration and Plate Count Methods.

RECOVERY METHOD	TAMC (# OF SCD PLATE[a])	TYMC (# OF SDA PLATE[a])	PRODUCT VOLUME (1:10) ML PER PLATE	INNOCULUM
Membrane Filtration				
Test group	1	1	10 (DF = 1)	Yes
Peptone control	1	1	0	Yes
Inoculum control	1	1	0	Yes
Product negative control	1	1	10 (DF = 1)	No
Negative control	1	1	0	No
Pour-Plate				
Test group	2	2	1 (DF = 10)	Yes
Peptone control	2	2	0	Yes
Inoculum control	2	2	0	Yes
Product negative control	2	2	1 (DF = 10)	No
Negative control	2	2	0	No
Spread-Plate				
Test group	2	2	0.1 (DF = 100)	Yes
Peptone control	2	2	0	Yes
Inoculum control	2	2	0	Yes
Product negative control	2	2	0.1 (DF = 100)	No
Negative control	2	2	0	No

[a]# of SCD plates in a single experiment
DF = dilution factor

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Address correspondence to Nicole Vu, PhD, Analytical Research Laboratories, Inc., 840 Research Parkway, Suite 546, Oklahoma City, OK 73104. E-mail: nvu@arlok.com