The Harmonization of the Microbial Limits Tests

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The *US Pharmacopeia (USP)*, the *Japanese Pharmacopoeia*, and the *European Pharmacopoeia* "Microbial Limits Tests" are in the final stages of harmonization. The harmonized *USP* chapters are slated for implementation in 2007. This article describes the harmonized *USP* Chapters ⟨61⟩ "Microbial Enumeration," ⟨62⟩ "Absence of Specified Microorganisms," and ⟨1111⟩ "Microbiological Attributes of Nonsterile Pharmaceutical Products," and suggests they will likely require some revalidation of existing methodologies. Companies should put plans in place immediately for this work and show consistent progress towards this goal.

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The *US Pharmacopeia* (*USP*) and the *European Pharmacopoeia* (*Pharm Eur*) "Microbial Limits Tests" are in the final stages of harmonization. They were signed off to Stage 6A at the November 2005 meeting of the Pharmacopeial Discussion Group held in Chicago, Illinois (1), and the harmonized versions have been published (see *USP* 2006, supplement 2). The harmonized chapters (Stage 6B, see sidebar, "Stages of the Pharmacopeial Discussion Group process") do not differ significantly from the drafts published in 2003 (2–4).

The format of the *USP* chapters changes dramatically with this harmonization. Whereas the Microbial Limits Tests were two chapters in the *USP 29* (5, 6), they are now modified in the harmonized version to mirror the European format (see Table I).

The implementation of the tests is on different schedules in the United States and in Europe. In the United States, the tests were originally scheduled to become effective Aug. 1, 2007, but the implementation date has been postponed to May 1, 2007 on the basis of comments received by USP. In Europe, implementation has three different schedules depending upon the situation:

1. Substances covered by a monograph specification: use the methods of the *European Pharmacopoeia* (A) until the monograph is revised and implemented (projected date: January 2009).

2. Substances not covered by a monograph specification: use either the methods of the *European Pharmacopoeia* (A) or the harmonised methods (B) until January 2010. From January 2010: use harmonised methods (B).

3. Preparations: use either method of the *European Pharmacopoeia* (A) or the harmonised method (B) of chapter 5.1.4 until January 2010. For new preparations, use of harmonised method (B) is advisable. From January 2010: Use harmonised method (B) of chapter 5.1.4 (7).

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Stages of the Pharmacopeial Discussion Group process

1. Identification of the item to be harmonized.
2. Investigation into existing texts.
3. Proposal for the Expert Committee Review.
4. Official inquiry
   This version is published in the *Pharmacopeial Forum* or *PharmEuropa* for public comment.
5. Consensus
   5A: Provisional
   This is the coordinating pharmacopeia's proposal for consensus. If all three agree, it moves to draft sign-off (5B). Otherwise, work continues for consensus.
   5B: Draft sign-off
6. Regional adoption and implementation.
   6A: Adoption
   6B: Implementation
7. Inter-regional implementation

Table I: Harmonized chapter numbers.

US Pharmacopeia	*European Pharmacopeia*
⟨61⟩ "Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests"	2.6.12 "Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests"
⟨62⟩ "Microbiological Examination of Nonsterile Products: Tests for Specified Microorganisms"	2.6.13 "Microbiological Examination of Nonsterile Products: Tests for Specified Microorganisms"
⟨1111⟩ "Microbiological Quality of Nonsterile Pharmaceutical Products"	5.1.4 "Microbiological Quality of Nonsterile Pharmaceutical Products"

USP ⟨61⟩ "Microbial Enumeration"

The microbial enumeration test is a basic, simple design to count the number of colony-forming units (CFUs) in a nonsterile product or raw material. The preferred method is to put the material into a solution and then plate the aliquots to determine the CFUs/g (or mL) of initial material. If the product cannot be put into a solution, the most probable number (MPN) method has several provisions to use. A full description of the MPN method is outside the scope of this article, but interested readers can refer to the discussion in the US Food and Drug Administration's *Bacterial Analytical Manual* (8).

The method of plating can be either pour plate, spread plate, or material filtration and then placing the membrane filter on an agar plate surface. The membrane filtration method should only be used when few CFUs are expected to be found in the material to be tested. Though membrane filtration is a good method to test a large volume of liquid, it can only count as many as 100 CFUs/membrane.

The harmonized method provides much more detail than any of the current pharmacopeial methods in terms of demonstrating method suitability (method validation) and media-growth promotion.

The demonstration of method suitability should be performed using the challenge organisms listed (see Tables IIa–b) in accordance with the recommendations found in *USP* Chapter ⟨1227⟩ (9). Growth promotion is an area of some ambiguity in the compendial text. Although media-growth promotion is not described in the tests, it is required. *USP* Chapter ⟨1117⟩ (10) provides assistance in designing the studies using 10–100 CFUs of the challenge organisms.

A major concern of many quality control workers is whether the changes in the harmonized chapter will necessitate the revalidation of existing assays to meet harmonized test requirements. Several considerations might lead to revalidation: a required change in media, in the volume of material required for testing, or in general testing conditions. It is difficult to determine whether all product types would require revalidation, and thus a summary table (see Tables IIa–b) is included in this article to describe the critical aspects of the current "Microbial Limits Tests (Enumeration)" and the draft harmonization text. This table is provided only as an aid. The decision of whether or not revalidation is necessary rests with each individual facility for its particular products.

USP ⟨62⟩ "Absence of Specified Microorganisms"

There is a significant controversy in the United States over the intent of this evaluation. FDA is bound by the concern expressed in the *Code of Federal Regulations* (21 *CFR* 211.113 and 21 *CFR* 211.165) relating to the importance of "objectionable microorganisms." This issue is addressed in the final section of this review because the harmonized Chapter ⟨1111⟩ deals with "other organisms."

Tables IIIa–c presents the existing "Microbial Limits—Absence of Specified Microorganisms" tests from the current *USP* and *Pharm Eur*, as well as the harmonized document. It is presented as an aid to evaluation, and may assist in determining whether revalidation of method suitability studies is needed. It should be noted that this harmonized chapter represents a true compromise by all parties, with (at least in the author's opinion) significant changes from the current *USP, Pharm Eur,* and *JP* chapters. Table IV provides guidance about the media-growth promotion expectations of the new chapters.

As the reader reviews these sections, it is reasonable to consider the current validation (or microbial recovery or method suitability) studies performed for currently marketed products. It is very likely that many of these studies may need to be performed again to meet the expectations of the harmonized procedure.

USP ⟨1111⟩ "Microbial Quality": a new compendial consideration of "other organisms"

Chapter ⟨1111⟩ "Microbial Examination of Nonsterile Products: Acceptance Criteria for Pharmaceutical Preparations and Substances for Pharmaceutical Use" is a relatively short section that has a significant impact. For the US reader, the

Table IIa: Aspects of the current and harmonized microbial limits tests (enumeration).

Issue	*US Pharmacopeia* ⟨61⟩	*European Pharmacopoeia* Chapter 2.6.12	Harmonized
Media-growth promotion, organisms for trypticase soy	• *Staphylococcus aureus* . • *Escherichia coli.* • *Pseudomonas aeruginosa.* • *Salmonella.*	• *S. aureus* ATCC 6538 (NCIMB 9518, CIP 4.83). • *E. coli* ATCC 8739 (NCIMB 8545, CIP 53.126). • *Bacillus subtilis* ATCC 6633 (NCIMB 8054, CIP 5262).	• *S. aureus* ATCC 6538 (NCIMB 9518, CIP 4.83, NBRC 13276). • *P. aeruginosa* ATCC 9027 (NCIMB 8626, CIP 82.118, NBRC 13275). • *B. subtilis* ATCC 6633 (NCIMB 8054, CIP 5262, NBRC 3134). Note: detail is provided on method of preparation and culture.
Media-growth promotion, organisms for Sabouraud dextrose	Not mentioned	• *Candida albicans* ATCC 10231 (NCPF 3179, IP 48.72). • *Aspergillus niger* ATCC 16404 (IMI 149007, IP 1431.83).	• *C. albicans* ATCC 10231 (NCPF 3179, IP 48.72, NBRC 1594). • *A. niger* ATCC 16404 (IMI 149007, IP 1431.83, NBRC 9455). Note: detail is provided on method of preparation and culture.
Media-growth promotion, methodology	Not detailed	Use less than 100 CFUs/media. Counts must be within 5-fold of control (95% confidence interval for MPN).	Use less than 100 CFU/media. Counts must be within 50% of control (95% confidence interval for MPN).
Media sterility check	Not detailed	Combined with negative product control.	Recommended.
Suitability of the counting method	• Inoculate diluted specimens of the product to be tested with challenge organisms. • May stop validation effort after sufficient effort has shown a particular organism cannot be recovered.	Must show recovery in presence of product.	• Preparation of test strains is detailed. • Use less than 100 CFU of the challenge organism. • Instruction is provided on the neutralization of antimicrobial activity. • Recovery must be within 50% of control. • May stop validation effort after sufficient effort has shown a particular organism cannot be recovered.
Sampling plan	Not detailed	Must follow well-defined sampling plan: • Batch size. • Health hazard. • Product characteristics. • Expected level of contamination.	Sampling plan is not detailed. Discussion of modifications to sample volume: • Active agents in low concentrations. • Bulk materials. • Small batches.
Sample volume	10 g	10 g	10 g
Categories	• Water soluble. • Water-immiscible fluids, ointments, creams, waxes. • Fluid in aerosol form.	• Water-soluble products. • Nonfatty products insoluble in water. • Fatty products. • Transdermal patches.	• Water-soluble products. • Nonfatty products insoluble in water. • Fatty products. • Fluids or solids in aerosol form. • Transdermal patches.
Methodology, membrane filtration	Not listed	• Transfer 1g to each of two filters (0.45 µm nominal pore size). • Three 100-mL washes. • One filter on TSA, one on SDA. • Incubate TSA plate at 30–35 °C for 5 days. • Incubate SDA plate at 20–25 °C for NMT 5 days. • Count plates with NMT 100 CFU.	• Transfer validated amount to two filters. • Wash each filter with a validated method. • TAMC filter is placed on TSB, incubated at 30–35 °C for 3–5 days. • TYMC filter is placed on SDA, incubated at 20–25 °C for 5–7 days.
Methodology, plate count: pour plate	• Add 1 mL of sample at appropriate dilution to a 9-cm diameter petri dish. Add 15–20 mL of liquified agar (TSA, SDA). • Plate in duplicate. • Incubate TSA at 30–35 °C for 18–72 h for TAMC; Incubate SDA at 20–25 °C for 5 days for total combined yeast and mold count.	• Add 1 mL of sample at appropriate dilution to a 9-cm diameter petri dish. Add 15–20 mL of liquified agar (TSA and SDA) • Plate in duplicate • Incubate as above • Count plates with not more than 300 CFU	• Prepare sample with a method shown to be suitable. • Plate 1 mL in at least duplicate on TSA and SDA. • Incubate TSA plates at 30–35 °C for 3–5 days. • Incubate SDA plates at 20–25 °C for 5–7 days. • Count from plates with less than 250 for TAMC, less than 50 CFU for TYMC.
Methodology, plate count: spread plate	Not listed	• Add 0.1 mL of sample at the appropriate dilution to 9-cm diameter petri dish containing agar (TSA and SDA) • Plate in duplicate • Incubate as above • Count plates with not more than 300 CFU	• Prepare sample by a method shown to be suitable. • Plate 0.1 mL in at least duplicate on TSA and SDA by spreading on surface of prepared plates. • Incubate as above. • Count from plates with less than 250 for TAMC, less than 50 CFU for TYMC.
Methodology, MPN	• Assemble 14 tubes of TSB (9 mL each). • Perform a 10-fold dilution series of the sample (in triplicate) into 12 tubes. • Incubate all 14 tubes. • Negative controls must be clear. • Read results from table.	• Use only for bacteria • Prepare at least three 10-fold dilutions in series • Inoculate three aliquots of each dilution 1-g or 1-mL samples into each of three tubes of 9–10 mL TSB • Incubate 30–35 °C for 5 days • Read results from table provided	• Prepare sample by a method shown to be suitable. • Incubate tubes at 30–35 °C for 3–5 days. • Read results from table provided.

Abbreviations: CFU is colony-forming unit, MPN is most probable number, NMT is not more than, SDA is Sabouraud dextrose agar, TAMC is total aerobic microbial count, TSA is trypticase soy agar, TSB is tryptic soy broth, and TYMC is total yeast and mold count.

Table IIb (continued): Aspects of the current and harmonized microbial limits tests (enumeration).

Issue	*US Pharmacopeia* ⟨61⟩	*European Pharmacopoeia* Chapter 2.6.12	Harmonized
Additional controls	None	Use sterile sodium chloride–peptone solution pH 7.0 as a test preparation to test: • Sterility of medium. • Sterility of diluent. • Aseptic performance of the test.	Use sterile diluent as the test preparation for each batch of diluent to verify testing conditions.
Interpretation of results	• Must meet specifications. • Retest allowed using 25-g sample.	Must be within five-fold of specification for product.	Must be within two-fold of specification for product.

allowance for twice the specification in observed results is significant. But, this is not the major change.

Before the introduction of the harmonized Chapter ⟨1111⟩, *USP* was only interested in specified organisms. These organisms are specified in monographs. But, FDA has been concerned about objectionable organisms. The "Control of Microbiological Contamination (a)" section of 21 *CFR* 211.113 states, "Appropriate written procedures, designed to prevent objectionable microorganisms on drug products not required to be sterile, shall be established and followed." This is reinforced by 21 *CFR* 211.165 which states in the section "Testing and release for distribution... (b) There shall be appropriate laboratory testing, as necessary, of each batch of drug product required to be free of objectionable microorganisms."

Thus, industry has had a problem. The *USP* monograph for a product (as provided in the current *National Formulary* [*NF*]) may require the "Absence of *Pseudomonas aeruginosa.*" A test in the "Microbial Limits" chapter demonstrates the absence of *P. aeruginosa.* Although this test may be needed to demonstrate compliance with the monograph requirements laid out in the *National Formulary*, it does not meet FDA's concern that all microorganisms in a nonsterile product should be acceptable to the product and the target population (*i.e.*, are not "objectionable").

The FDA concern

If a company's product approval to market submission states it will test the finished product by the "Microbial Limits Tests," FDA will enforce the good manufacturing practices (GMPs) requirement that it must do so. This is purely a GMP concern. Nonetheless, the agency has been absolutely clear about its concern over objectionable microorganisms in the product and that testing to the *USP* chapter might be necessary, but it is not sufficient to demonstrate acceptable microbial quality. In fact, in the 1993 instructional guide for inspections of quality-control microbiology laboratories (11), FDA points out several issues that have occurred by particular contaminants of nonsterile medicines that compromised patient health. The document notes that the *USP* provides methods for specific organisms, but not all objectionable organisms and FDA strongly recommends all organisms be identified to determine which are acceptable and which are objectionable. This section of the guidance concludes:

> The importance of identifying all isolates from either or both Total Plate Count testing and enrichment testing will depend upon the product and its intended use. Obviously, if an oral solid dosage form such as a tablet is tested, it may be acceptable to identify isolates when testing shows high levels. However, for other products such as topicals, inhalants or nasal solutions where there is a major concern for microbiological contamination, isolates from plate counts, as well as enrichment testing, should be identified.

Why is microbial contamination of concern? To understand, we must consider the history on this matter. As early as 1942, *USP* had a test for the "Bacteriological Examination of Gelatin" (12). But, most nonsterile medications in the United States were not required to assay for microbiological quality attributes until the introduction of the "Microbial Limits Tests" in 1970 (13). In the late 1960s, several outbreaks of disease were traced back to pathogen-contaminated medications, which prompted increased attention to the microbial content of nonsterile drugs (14). Later in the 1980s, a series of articles described contamination by *P. cepacia* (currently *Burkholderia cepacia*) (15, 16) and its survival in disinfectants (17–21). This concern led to the addition of requirements in the 21 *CFR* to ensure that no objectionable organisms are in product released to market.

The compendial concern

As early as 1982, the *USP* is on record for verifying that the demonstration of "absence of objectionable microorganisms" is not the intent of the chapter. In a one-page Stimuli to the Revision Process (22), the microbiology committee of the time states:

> The tests described in the Microbial Limits Tests ⟨61⟩ were not designed to be all-inclusive, i.e., to detect all potential pathogens. To accomplish this, an extensive text on laboratory detection of microorganisms would be required. The procedures in USP were designed to detect the presence of specific "index" or "indicator" organisms. Nevertheless, the present chapter does not preclude the detection of *Ps. Cepacia* - the organism requires subsequent differentiation. The chapter does not provide specific methods for this, nor does it provide procedures for detecting thousands of other potentially pathogenic organisms. Individual monographs include requirements for limits on total aerobic counts and/or absence of one or more of the four selected "indicator" organisms. The chapter on Microbial Limits Tests provides methods to assure that one may test for those microbial requirements in the individual monographs.

Table IIIa: Absence of specified microorganisms.

Issue	*US Pharmacopeia* ⟨61⟩ (current)	*European Pharmacopoeia* Chapter 2.6.13 (current)	Harmonized
Media-growth promotion, organisms for trypticase soy	• *Staphylococcus aureus.* • *Escherichia coli.* • *Pseudomonas aeruginosa.* • *Salmonella.*	• *S. aureus* ATCC 6538 (NCIMB 9518, CIP 4.83). • *E. coli* ATCC 8739 (NCIMB 8545, CIP 53.126). • *Salmonella typhimurium* (a strain not pathogenic for man is to be used). • *P. aeruginosa* ATCC 9027 (NCIMB 8626, CIP 82.118).	• *S. aureus* ATCC 6538 (NCIMB 9518, CIP 4.83, NBRC 13276). • *P. aeruginosa* ATCC 9027 (NCIMB 8626, CIP 82.118, NBRC 13275). • *E. coli* ATCC 8739 (NCIMB 8545, CIP 53.126. NBRC 3972). • *Salmonella enterica spp typhimurium* ATCC 14028 or *Salmonella enterica spp abony* NCTC 6017 of CIP 80.39. • *Candida albicans* ATCC 10231 (NCPF 3179, IP 48.72, NBRC 1594) (for SDA). Note: detail is provided on method of preparation and culture.
Media-growth promotion, methodology	Not detailed.	Use less than 100 CFU/media in mixture.	• Detailed instructions for the confirmation of nutritive, selective, and indicative properties of media (see Table IV). Colonies are comparable in appearance and indication reactions to a previously used batch of medium. • Use less than 100 CFU.
Method suitability		• Use less than 100 CFU/media in mixture. • All tests must work in the presence and absence of the product	Detailed instructions for the confirmation of nutritive, selective, and indicative properties of media in the presence of the product. (see Table IV).
Media sterility check	Not detailed.	Combined with negative product control.	Recommended.

Test for *Staphylococcus aureus*	• Bring specimen up to 100 mL with TSB.
	• Incubate.
	• If growth, streak on Vogel–Johnson agar medium (or Baird Parker agar, or mannitol–salt agar.
	• Compare colonies for characteristics given. If absent, meets specification.
	• If suspect colonies present, conduct coagulase test. Must be coagulase negative to meet specifications.
		• Bring specimen up to 100 mL with TSB and incubate at 35–37 °C for 18–48 h.
		• Streak onto Baird-Parker agar and incubate at 35–37 °C for 18–72 h.
		• Examine for black colonies of gram-positive cocci.
		• Confirm by biochemical tests.
		  • Coagulase.
		  • Deoxyribonuclease test.
			Sample Preparation
			• Inoculate a suitable amount of TSB with 1 g of sample. Incubate at 30–35 °C for 18–24 h.
			Selection and Subculture
			• If growth, streak onto mannitol–salt agar and incubate at 30–35 °C for 18–72 h.
			• Examine colonies for distinctive morphology.
			• Confirm identity of suspect colonies.

Test for *Pseudomonas aeruginosa*	• Bring specimen up to 100 mL with TSB. • Incubate. • If growth, streak on a cetrimide agar medium. • Compare colonies for characteristics given. If absent, meets specification. • If suspect colonies present, streak colonies onto *Pseudomonas* agar medium for the detection of fluorescein and *Pseudomonas* Agar for the detection of pyocyanin. • Compare colonies for characteristics given on these additional agars. If absent, meets specification. • Confirm suspect colonies with oxidase test. Must be oxidase negative to meet specifications.	• Bring specimen up to 100 mL with TSB. Incubate at 35–37 °C for 18–48 h • If growth, streak onto cetrimide agar and incubate at 35–37 °C for 18–72 h. Product passes if there is no growth. • If growth, gram stain. If gram-negative rods seen, transfer colonies to TSB and incubate at 41–43 °C for 18–24 h. If no growth, the product passes.	**Sample Preparation** • Inoculate a suitable amount of TSB with 1 g of sample. Incubate at 30–35 °C for 18– 24 h. **Selection and Subculture** • If growth, streak onto cetrimide agar and incubate at 30–35 °C for 18–72 h. • Examine colonies for distinctive morphology. • Confirm identity of suspect colonies.

Abbreviations: CFU is colony-forming unit, TSB is tryptic soy broth.

Against this background, we now examine the short harmonized Chapter ⟨1111⟩, which consists of two tables and a few paragraphs. A significant passage in this chapter reads:

> In addition to the microorganisms listed in Table I [Table I is entitled "Acceptance Criteria for Microbiological Quality of Nonsterile Dosage Forms"], the significance of other microorganisms recovered should be evaluated in terms of the following:
>
> - The use of the product: hazard varies according to the route of administration (eye, nose, respiratory tract).
> - The nature of the product: does the product support growth? does it have adequate antimicrobial preservation?
> - The method of application.
> - The intended recipient: risk may differ for neonates, infants, the debilitated.
> - Use of immunosuppressive agents, corticosteroids.
> - The presence of disease, wounds, organ damage.

Table IIIb (continued): Absence of specified microorganisms.

Issue	*US Pharmacopeia* ⟨61⟩ (current)	*European Pharmacopoeia* Chapter 2.6.13 (current)	Harmonized

Test for *Salmonella spp*	• Bring specimen up to 100 mL with fluid lactose medium.
	• Incubate.
	• If growth, pipette 1 mL into 10 mL:
	  • fluid selenite medium.
	  • fluid tetrathionate medium.
	• Incubate 12–24 h.
	• Streak growth of both fluid selenite and fluid tetrathionate onto:
	  • brilliant green agar.
	  • xylose–lysine–deoxycholate agar.
	  • bismuth–sulfite agar.
	• Incubate for growth. Examine colonies for characteristic morphology.
	• For colonies with characteristic morphology seen, examine for gram-negative rods.
	• Stab-streak colonies with gram-negative rods into a butt-slant of triple sugar–iron–agar. Incubate the slants and examine for red slants with yellow butts. If seen, product fails specification.
		• Subculture on at least two agars:
		  • deoxycholate citrate agar.
		  • xylose–lysine–deoxycholate agar.
		  • brilliant green, phenol red, lactose–monohydrate–sucrose agar.
		• Examine colonies for distinctive morphology.
		• Stab-streak colonies with gram-negative rods into a butt-slant of triple sugar–iron–agar. Incubate the slants and examine for red slants with yellow butts.
		• Confirmation may be done using biochemical and serological tests.
			Enrichment broth. Incubate at 30–35 °C for 18–24 h.
			• If growth, streak onto xylose–lysine–deoycholate agar.
			• Examine colonies for distinctive morphology.
			• Confirm identity of suspect colonies.

Test for *Escherichia coli*	• Bring specimen up to 100 mL with fluid lactose medium. • Incubate. • If growth, streak onto MacConkey agar medium. • Incubate and then examine for distinctive colony morphology. • If colony morphology seen, transfer suspect colonies to levine eosin–methylene blue agar medium and streak for single colonies. • Incubate and examine for distinctive colony morphology. If seen, product fails specification.	• Bring specimen up to 100 mL with TSB and incubate at 35–37 °C for 18–48 h. • Transfer 1 mL to 100 mL MacConkey broth and incubate at 35–37 °C for 18–72 h. • Streak onto MacConkey agar and incubate at 35–37 °C for 18-48 h. • If distinctive colony morphology seen, confirm with suitable tests, such as indole production.	• Inoculate a suitable amount of TSB with 1 g of sample. Incubate at 30–35 °C for 18– 24 h. • If growth, transfer 1 mL to 100 mL MacConkey medium. Incubate at 42–44 °C for 24–48 h. • If growth, streak onto MacConkey agar and incubate at 30–35 °C for 18–72 h. • If no growth, or if confirmatory tests show absence of *E. coli*, product passes.
Test for bile-tolerant gram-negative bacteria	None	None	**Sample preparation** • Suspend not less than 1 gram sample in TSB, incubate at 20–25 °C for 2–5 h. **Test for absence** • Use the volume corresponding to the limit required to inoculate a suitable amount of Mossel enterobacteriaceae enrichment broth. Incubate at 30–35 °C for 24–48 h. • If growth, streak onto violet–red bile–glucose agar medium and incubate at between 30–35 °C for 18–24 h. • Product passes if no red colonies surrounded by a reddish precipitate. **Quantitative test** • Use the sample prepared above under "Sample Preparation" corresponding to 10–1, 10–2, 10–3 dilutions into Mossel enterobacteriaceae enrichment broth. Incubate at 30–35 °C for 24–48 h. • Plate samples onto violet–red bile glucose agar medium. Incubate for growth. • Note the smallest quantity of the product that gives growth.

Abbreviations: CFU is colony-forming unit, TSB is tryptic soy broth.

Table IIIc (continued): Absence of specified microorganisms.

Issue	*US Pharmacopeia* ⟨61⟩ (current)	*European Pharmacopoeia* Chapter 2.6.13 (current)	Harmonized
Test for enterobacteria and certain other gram-negative bacteria	None	**Detection** • Suspend sample in Lactose monohydrate broth, incubate at 35–37 °C for 2–5 h. • Transfer contents equivalent to 1-g product to 100-mL enterobacteria enrichment broth Mossel. Incubate for 18–24 h. Passes if no growth. **Quantitative evaluation** • Dilute the lactose–monohydrate broth suspension 10–1, 10–2, 10–3 into enterobacteria enrichment broth-Mossel. Incubate for 24–48 h. • Plate samples onto crystal violet, neutral red, bile agar with glucose media. Incubate for growth. • Note the smallest quantity of the product that gives growth.	None
Sample volume	10 g	10 g	1 g
Test for *Clostridia*	None	Specific tests for the presence of *Clostridia* and for the enumeration of *Clostridia perfringens*	Specific tests for the presence of *Clostridia*
Test for *Candida albicans*	None	None	• Inoculate a suitable amount of Sabouraud dextrose broth with 1 g of sample. Incubate at 20–25 °C for 5–7 days. • If growth, streak onto a Sabouraud dextrose agar Incubate at 20–25 °C for 2 days. • Examine colonies for distinctive morphology. • Confirm identity of suspect colonies. If no growth, or if confirmatory tests show absence of *C. albicans*, product passes.
Additional controls	None	Use sterile sodium chloride–peptone solution (pH 7.0) as test preparation to test: • Sterility of medium. • Sterility of diluent. • Aseptic performance of the test.	Use sterile diluent as the test preparation for each batch of diluent to verify testing conditions.
Interpretation of results	• Must meet specs. • Retest is allowed using 25-g sample.	• Must meet specs. • No retest.	• Must meet specs. • No retest.

Where warranted, a risk-based assessment of the relevant factors is conducted by personnel with specialized training in microbiology and in the interpretation of microbiological data. For raw materials, the assessment takes account of the processing to which the product is subjected, the current technology of testing, and the availability of materials of the desired quality.

The harmonized chapter does not provide instruction beyond this level, but do not overlook this "new" recommendation. Merely showing the absence of specified organisms is not sufficient to demonstrate the microbial quality of a nonsterile product.

Conclusions

The US Pharmacopeia and the US Food and Drug Administration are in agreement about the question of the microbial quality of nonsterile pharmaceuticals: the product must be safe for use. The internationally harmonized chapters provide a strong framework for this assurance.

The introduction of these three harmonized chapters is likely to require some revalidation of existing methodologies. Companies should put plans in place immediately for this work and show consistent progress toward this goal.

The *National Formulary* monograph requirements for the absence of specific organisms is a minimal requirement and should not be taken as proof that the product is suitable for sale from a microbiological perspective. Harmonized Chapter ⟨1111⟩ recommends the determination of the risk associated with "other organisms," which is in agreement with the FDA expectation for absence of "objectionable" organisms. The manufacturer is responsible for the quality and safety of the product marketed, and it is FDA's clear expectation (as described in *CFR*) that this will include a determination of the microbial safety (*i.e.*, the "absence of objectionable microorganisms") from the product. These positions have been publicly stated for decades and should not come as a surprise. The harmonized microbial limits tests only address the "absence of specified microorganisms" and leave the determination of the "absence of objectionable microorganisms" in the capable hands of each company's appropriately educated and well-trained microbiology group.

References

1. USP Chapter ⟨1196⟩, "Pharmacopeial Harmonization," *US Pharmacopeia (USP) 29–National Formulary (NF) 24* (US Pharmacopeial Convention [USP], Rockville, MD, 2006), pp. 3031–3035.
2. USP, Chapter ⟨61⟩, "Microbiological Examination Of Nonsterile Products: Microbial Enumeration Tests," *Pharm Forum.* **29** (5), 1714–1722 (2003).

Table IV: Nutritive, selective, and indicative properties of media.

Medium	Property	Test strains
Test for bile-tolerant gram-negative bacteria		
Mossel enterobacteriaceae enrichment broth	nutritive	*Escherichia coli, Pseudomonas aeruginosa*
	selective	*Staphylococcus aureus*
Violet–red bile glucose agar medium	nutritive, indicative	*E. coli, P. aeruginosa*
Test for *E. coli*		
MacConkey medium	nutritive	*E. coli*
	selective	*S. aureus*
MacConkey agar medium	nutritive, indicative	*E. coli*
Test for *Salmonella*		
Rappaport Vassiliadis *Salmonella* enrichment broth	nutritive	*Salmonella enterica spp. typhimurium* (or *S. enterica spp. abony*), *E. coli*
Xylose-lysine-deoxycholate agar	nutritive, indicative	*Salmonella enterica spp. typhimurium* (or *S. enterica spp. abony*), *E. coli*
Test for *P. aeruginosa*		
Cetrimide agar medium	nutritive	*P. aeruginosa*
	selective	*E. coli*
Test for *S. aureus*		
Mannitol–salt agar medium	nutritive	*S. aureus*
	selective	*E. coli*
Test for *Clostridia*		
Reinforced medium for *Clostridia*	nutritive	*C. sporogenes*
Columbia agar medium	nutritive	*C. sporogenes*
Test for *Candida albicans*		
Sabouraud–dextrose medium	nutritive	*C. albicans*
Sabouraud–dextrose agar medium	nutritive, indicative	*C. albicans*

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