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The Microbiological Requirements of a Stability Study

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Introduction

- Therapeutic Goods Order no 77 Microbiological standards for medicines.
- Harmonized British, European and United States Pharmacopoeia.
- At a minimum, testing should be conducted at the initial and end time points of a stability study.

Harmonized Test Methods

- **British Pharmacopoeia**, Appendix XVI, D Microbiological Quality of Pharmaceutical Preparations B. HARMONISED METHOD: MICROBIOLOGICAL QUALITY OF NON-STERILE PHARMACEUTICAL PREPARATIONS AND SUBSTANCES FOR PHARMACEUTICAL USE, when tested by the methods of:
- (i) the British Pharmacopoeia, Appendix XVI B. Test for Microbial Contamination 2. Total viable aerobic count. B. HARMONISED METHOD: MICROBIOLOGICAL EXAMINATION OF NON-STERILE PRODUCTS : MICROBIAL ENUMERATION TESTS, and
- (ii) the British Pharmacopoeia, Appendix XVI B. Test for Microbial Contamination 1. Tests for specified micro-organisms B. Harmonised method; or
- (b) the European Pharmacopoeia, B: Harmonised Method: Microbiological Quality Of Non-Sterile Pharmaceutical Preparations And Substances For Pharmaceutical Use (5.1.4); when tested by the methods of:
- **European Pharmacopoeia**, Harmonised Method: Microbiological Examination of Non-sterile Products: Microbial Enumeration Tests (2.6.12); and
- **European Pharmacopoeia**, Harmonised Method: Microbiological Examination of Non-sterile Products: Test for Specified Micro-organisms (2.6.13); or
- **United States Pharmacopoeia** – National Formulary, chapter <1111> , MICROBIOLOGICAL EXAMINATION OF NONSTERILE PRODUCTS: ACCEPTANCE CRITERIA FOR PHARMACEUTICAL PREPARATIONS AND SUBSTANCES FOR PHARMACEUTICAL USE, when tested by the methods of:
- (i) the United States Pharmacopoeia – National Formulary, chapter <61> MICROBIOLOGICAL EXAMINATION OF NONSTERILE PRODUCTS: MICROBIAL ENUMERATION TESTS; and
- (ii) the United States Pharmacopoeia – National Formulary, chapter <62> MICROBIOLOGICAL EXAMINATION OF NONSTERILE PRODUCTS: TESTS FOR SPECIFIED MICROORGANISMS.

- **Tests include Total aerobic microbial count, Total yeast and mould count, Bile Tolerant Gram Negative bacteria, *E. coli*, Salmonella, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, Clostridia and *Candida albicans*.**

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Acceptance Criteria

Table 5.1.4.-1. – *Acceptance criteria for microbiological quality of non-sterile dosage forms*

Route of administration	**TAMC (CFU/g or CFU/mL)**	**TYMC (CFU/g or CFU/mL)**	**Specified micro-organisms**
Non-aqueous preparations for oral use	10^3	10^2	Absence of *Escherichia coli* (1 g or 1 mL)
Aqueous preparations for oral use	10^2	10^1	Absence of *Escherichia coli* (1 g or 1 mL)
Rectal use	10^3	10^2	-
Oromucosal use Gingival use Cutaneous use Nasal use Auricular use	10^2	10^1	Absence of *Staphylococcus aureus* (1 g or 1 mL) Absence of *Pseudomonas aeruginosa* (1 g or 1 mL)
Vaginal use	10^2	10^1	Absence of *Pseudomonas aeruginosa* (1 g or 1 mL) Absence of *Staphylococcus aureus* (1 g or 1 mL) Absence of *Candida albicans* (1 g or 1 mL)
Transdermal patches (limits for one patch including adhesive layer and backing)	10^2	10^1	Absence of *Staphylococcus aureus* (1 patch) Absence of *Pseudomonas aeruginosa* (1 patch)
Inhalation use (special requirements apply to liquid preparations for nebulisation)	10^2	10^1	Absence of *Staphylococcus aureus* (1 g or 1 mL) Absence of *Pseudomonas aeruginosa* (1 g or 1 mL) Absence of bile-tolerant gram-negative bacteria (1 g or 1 mL)
♦Special Ph. Eur. provision for oral dosage forms containing raw materials of natural (animal, vegetal or mineral) origin for which antimicrobial pretreatment is not feasible and for which the competent authority accepts TAMC of the raw material exceeding 10^3 CFU/g or CFU/mL.	10^4	10^2	Not more than 10^2 CFU of bile-tolerant gram-negative bacteria (1 g or 1 mL) Absence of *Salmonella* (10 g or 10 mL) Absence of *Escherichia coli* (1 g or 1 mL) Absence of *Staphylococcus aureus* (1 g or 1 mL)♦

Acceptance Criteria

complementary medicine oral dosage form containing raw material of natural (animal, vegetal or mineral) origin

Microbiological Quality	Acceptance Criteria
Total aerobic microbial count	Less than or equal to 10^4 CFU per g or per mL
Total yeast and mould count	Less than or equal to 10^2 CFU per g or per mL
Bile-tolerant Gram negative bacteria	Less than or equal to 10^2 CFU per g or per mL
Salmonella	absent in 10 g or 10 mL
Escherichia coli	absent in 1 g or 1 mL
Staphylococcus aureus	absent in 1 g or 1 mL

Acceptance Criteria

- complementary medicine oral dosage form containing raw material of natural (vegetal) origin that is a herbal medicinal product consisting solely of one or more herbal substances (whole, reduced or powdered) to which boiling water is added before use

Microbiological Quality	Acceptance Criteria
Total aerobic microbial count	Less than or equal to 10^7 CFU per g
Total yeast and mould count	Less than or equal to 10^5 CFU per g
Bile-tolerant Gram negative bacteria	Less than or equal to10^2 CFU per g
Escherichia coli	absent in 1 g
Salmonella	absent in 10 g

Why PE Testing?

- British Pharmacopoeia Appendix XVI C - Efficacy of Antimicrobial Preservation
- European Pharmacopoeia, Section 5.1.3 Efficacy of Antimicrobial Preservation.
- United States Pharmacopoeia - chapter 51 Antimicrobial Effectiveness Test for Category 4 products.

- Key Factors affecting the efficacy of the antimicrobial preservative added:
    1. The active ingredient
    2. The excipients
    3. Storage conditions
    4. The container and its closure

- The BP states for a product "*it shall be demonstrated that the antimicrobial activity of the preparation as such or if necessary, with the addition of a suitable preservative or preservatives provides adequate protection from adverse effects that may arise from microbial contamination or proliferation during storage and use of the preparation*"

Selection of Category and Method

- All products sent for any PE testing after the prescribed stability storage conditions and times are recommended to be sent in the final packaged product as distributed by the sponsor.
- The BP/EP categories are for:
  - Parenteral, ophthalmic preparations, intramammary preparations
  - Ear preparations, nasal preparations, preparations for cutaneous application or inhalation.
  - Oral preparations, oromucosal and rectal preparations

Selection of Category and Method

- The preparation is individually challenged with a prescribed inoculum of 10^5 to 10^6 cfu/g/ml of preparation of bacteria and fungi and tested over 28 days.
- *Pseudomonas aeruginosa* ATCC 9027
- *Staphylococcus aureus* ATCC 6538
- *Candida albicans* ATCC 10231
- *Aspergillus brasiliensis* (formerly niger) ATCC 16404
- *Escherichia coli* ATCC 8739 for oral products
- *Zygosacharomyces rouxii* (NCYC 381) for high sugar oral products

Selection of Category and Method

- These are tested at the initial time points and various time intervals depending on the product category.
- These are performed by traditional plate count methods or membrane filtration.
- The test method must be qualified for the product under evaluation to ensure the correct diluent is used in assays for surviving microorganisms.

Selection of Neutralizer

ACTIVE ANTIMICROBIAL	METHOD OF INACTIVATING/NEUTRALISER
Alcohols	Dilution (+ Tween 80)
Aldehydes	3% Tween 80 + 0.3% Lecithin + 0.1 % L-histidine
Antibiotics	Membrane Filtration
Benzoic acid & its salts	Dilution + Tween 80
Boric acid (0.5%) Benzyl alcohol	1 % Tween 80 formulation or T3
Benzyl alcohol (0.3%)	5 % Tween 80 formulation or T6
Chlorhexidine	Tween 80 + Lecithin
Cresols	Dilution + Tween 80
Dowicil 200 (0.1%)	14 % Tween 80 formulation , T6, CAP IV
Kathon CG (0.03%)	10 % Tween 80 + 1 % Lecithin + 1 % Peptone, T6, CAP IV
Germall (0.2%)	5% Tween 80 formulation, T6.
Methyl paraben (0.1%)	
Propyl paraben (0.1%)	
Hypochlorites/Chloramino T/	0.5 % Sodium thiosulphate
Halogens derivatives	PICC = PCD + 0.1% bovine serum albumin
Mercury – base / heavy metals	0.05 – 0.5 % sodium thiosulphate
	0.08 – 0.15 % L-cysteine
	0.075 % Thiomalic acid (pH 7.0 with NaOH)
Peroxides	Catalase + Peroxydase (mix 1.4 mol H_2O_2- min. pH7/25^{0}C)
Phenolics	3 % Tween 80+0.3 % lecithin + 0.4 % Na-lauryl sulphate PCD = 3 % Tween 80 + 0.3 % Lecithin + 0.1 % L-histidine + 0.5 % sodium thiosulphate.
Quartenary Ammonium	3 % Tween 80 + 0.3 % lecithin (T3)
Sorbic acid	Dilution+Tween 80 or T3.

Acceptance Criteria

- Parenteral and ophthalmic preparations

		6h	24h	7day	14day	28day
Bacteria	A	2	3	-	-	No recovery
	B	-	1	3	-	No increase
Fungi	A	-	-	2	-	No increase
	B	-	-	-	1	No increase

- Ear, nasal, inhalation preparations and cutaneous application

		2day	7day	14day	28day
Bacteria	A	2	3	-	No increase
	B	-	-	3	No increase
Fungi	A	-	-	2	No increase
	B	-	-	1	No increase

Acceptance Criteria

- Oral, Oromucosal and Rectal Preparations

	14day	28day
Bacteria	3	No increase
Fungi	1	No increase

- For oral products of antacids with an aqueous base the above criteria must be met, however the initial inoculum must be between 1×10^3 and 1×10^4 cfu/ml of product tested.

BP and USP comparison

- Parenteral and ophthalmic preparations

		6h	24h	7day	14day	28day
Bacteria	A	2	3	-	-	No recovery
	B	-	1	3	-	No increase
USP		-	-	1.0	3.0	No increase from 14d
Fungi	A	-	-	2	-	No increase
	B	-	-	-	1	No increase
USP		-	-	NI	NI	NI

- Ear, nasal, inhalation preparations and cutaneous application,

		2day	7day	14day	28day
Bacteria	A	2	3	-	No increase
	B	-	-	3	No increase
USP				2	No increase from 14d
Fungi	A	-	-	2	No increase
	B	-	-	1	No increase
USP		-	-	NI	NI

BP and USP comparison

- Oral, Oromucosal and Rectal Preparations

	14day	28day
Bacteria	3	No increase
USP	1.0	No increase from 14d
Fungi	1	No increase
USP	NI	NI

Working Example

Mouthwash!

Working Example

- **Chlorohexidine Mouthwash**

1. Specific BP Monographs: Testing requirements outlined for chemical analysis.
2. The mouthwash complies with the requirements stated under Oromucosal Preparations.
3. General Monographs for Oromucosal Preparations:

   "A suitable test method together with the criteria for judging the preservative properties of the formulation are provided in 5.1.3 Efficacy of antimicrobial preservation .... In the manufacture, packaging, storage and distribution of oromucosal preparations, suitable means are taken to ensure their microbiological quality; recommendations on this aspect are provided in the text on Microbiological quality of pharmaceutical preparations "

Working Example

4. Microbiological testing requirements:

Oromucosal Use

Microbiological Quality	Acceptance Criteria
Total aerobic microbial count	Less than or equal to 10^2 CFU per ml
Total yeast and mould count	Less than or equal to 10^1 CFU per ml
Staphylococcus aureus	Absent per 1 ml
Pseudomonas aeruginosa	Absent per 1 ml

Working Example

- 5. Efficacy of Antimicrobial Preservation (PET)

  Oromucosal preparation

	LOG Reduction	
	14 day	28 day
Bacteria	3	No Increase
Fungi	1	No Increase

Working Example

- 5. Time points to be tested: initial, middle, end.
- 6. Storage conditions: temperature and humidity
- 7. Test method suitability by testing facility
- 8. Absence of Objectionable organisms
- 9. Interpretation of results.

Environmental Isolates

- Products may be more susceptible to environmental contamination during manufacturing due to the nature of the product and preservative system such as low pH or high water content.

- Although *Ps. aeruginosa* is included as a test organism, the reference culture strain may not be as resistant to the preservative system as various strains of *Enterobacter, Klebsiella* or *Burkholderia*.

- Manufacturing facilities with known environmental contaminants are recommended to isolate and include these strains in the PE testing.

Case Study 1

- The product was found to have high levels Total Aerobic Plate count levels exceeding the client specification.

- Identification of the isolated contaminant by both API and RiboPrinter™ found it to be *Enterobacter gergoviae*.

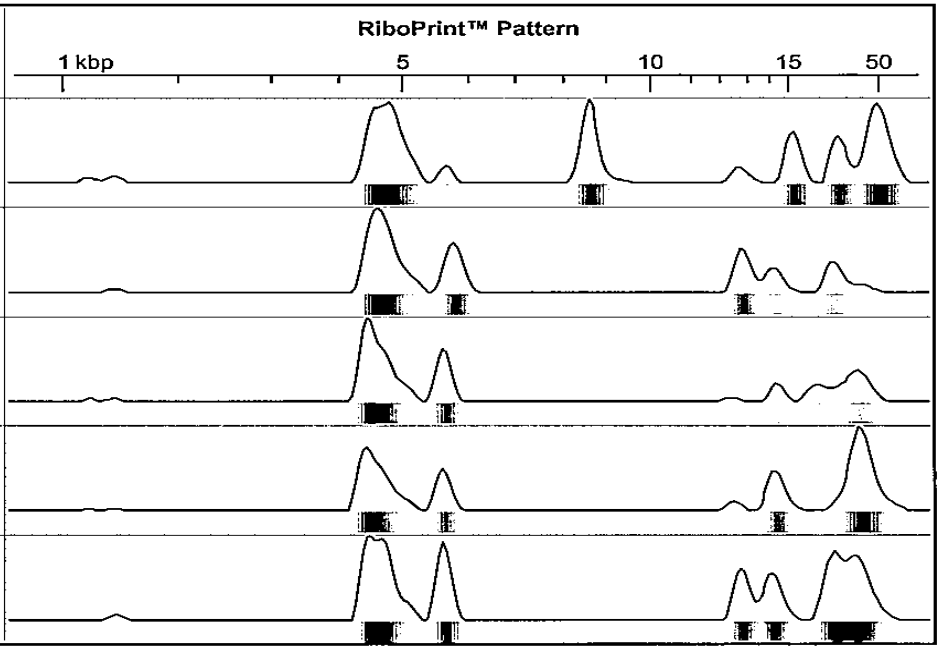
- Comparison of the strain through the RiboPrinter™ database found the strain to be unique for that facility.

Case Study 1

RiboPrinter® Microbial Characterization System
DuPont ID Report

DuPont Qualicon

	DuPont ID	Label	RiboPrint™ Pattern
1	DUP-16581	Enterobacter gergoviae	
2	DUP-19330	Enterobacter gergoviae	
3	DUP-10172	Enterobacter gergoviae	
4	DUP-15324	Enterobacter gergoviae	
5	DUP-18779	Enterobacter gergoviae	

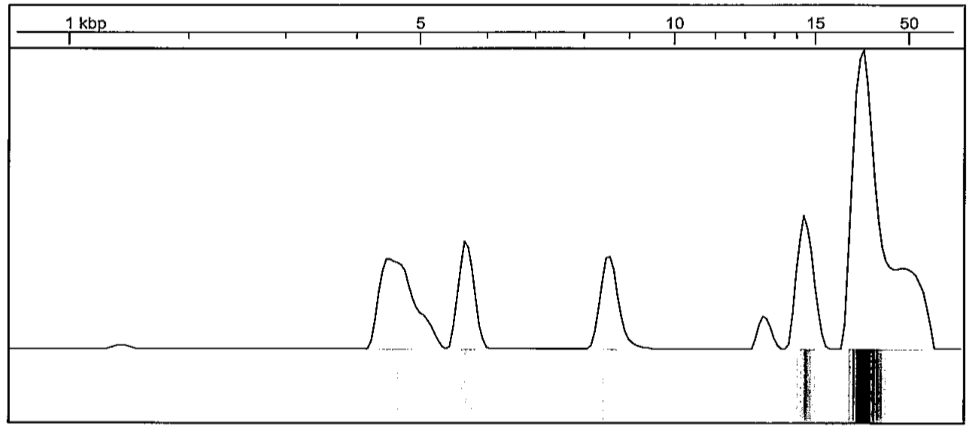


Case Study 1

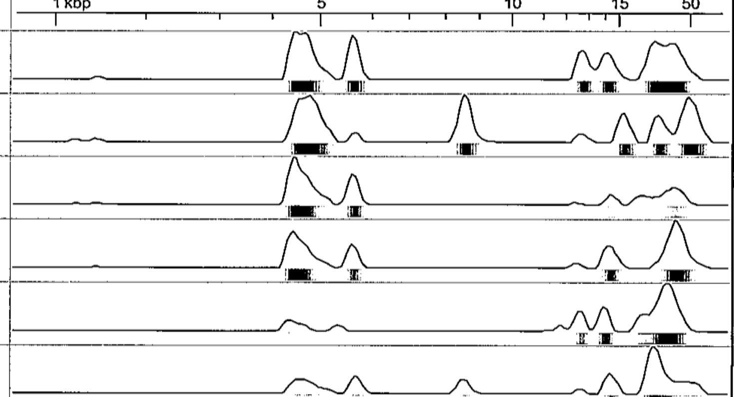
RiboPrinter® Microbial Characterization System
Sample 216-359-S-7 Report

DuPont Qualicon

Process	KHA
Enzyme	EcoRI
Label	PU0801820
Sample Comment	
Event Log	



	Type	Number	Similarity	Label	RiboPrint™ Pattern
1	DuPont Neighbor	DUP-18779	0.80	Enterobacter gergoviae	
2	DuPont Neighbor	DUP-16581	0.77	Enterobacter gergoviae	
3	DuPont Neighbor	DUP-10172	0.74	Enterobacter gergoviae	
4	DuPont Neighbor	DUP-15324	0.72	Enterobacter gergoviae	
5	DuPont Neighbor	DUP-6617	0.68	Vibrio parahaemolyticus	
6	RiboGroup	ECORI 216-359-S-7	1.00		



Not Approved for Clinical Diagnosis

Monday, April 26, 2010 2:49 PM

Case Study 1

- AMS Labs performed PE testing with the addition of the isolated *E. gergoviae* strain.
- Results from the PE test showed no recovery of all four test organisms, however, the *E. gergoviae* counts far exceeded those of the reference cultures.
- This was found to be attributed to the lower pH of the product and the natural paraben resistance *E. gergoviae*.

Case Study 1

Time Point	*S. aureus* AMS 027 (ATCC6538)	*P.aeruginosa* AMS 095 (ATCC 9027)	*C.albicans* AMS 003 (ATCC 10231)	*A.niger* AMS 032 (ATCC 16404)	*Enterobacter gergoviae*
Inoculum cfu/ml	7.5×10^5	5.4×10^5	1.0×10^6	1.6×10^5	9.1×10^5
0 hr	9.3×10^5	5.4×10^5	8.7×10^5	1.6×10^5	4.9×10^5
48 hr	<10	<10	N/A	N/A	6.4×10^2
7 days	<10	<10	N/A	N/A	4.4×10^2
14 days	<10	<10	<10	1.0×10^2	1.1×10^4
28 days	<10	<10	<10	<10	$>2.5 \times 10^6$

All results are expressed as CFU (Colony Forming Unit) per g. < = Less than > = greater than
N/A = Not Applicable

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Case Study 1

- 3 other products – all of them topical preparations were found to show increased growth in *E. gergoviae* over the 28 day test.

- All results showed slight reductions in counts after 48 hours from the preservative system, however, resistance was demonstrated by increases in counts after 14 days with only one out of the four products tested showing a reduction in counts at day 28 from the 14 day count.

Case Study 2

- Customer Complaint Product of shampoo
- Identification of contaminants by Vitek 2 found *Aeromonas hydrophila / caviae* 98% Excellent ID and *Pseudomonas putida* 99% Excellent ID
- Initial PET results met all criterias for topical/cutaneous preparations.
- Isolation and preservation of isolates to include in PET testing of 3 month product stored at room temperature.

Case Study 2

Results from PE Testing at initial timepoint; immediately after manufacture.

Time Point	*S. aureus* AMS 027 (ATCC6538)	*P.aeruginosa* AMS 095 (ATCC 9027)	*C. albicans* AMS 003 (ATCC 10231)	*A.niger* AMS 032 (ATCC 16404)
Inoculum cfu/ml	9.1×10^5	8.6×10^5	9.8×10^5	3.2×10^5
0 hr	9.3×10^4	5.8×10^5	1.2×10^6	2.4×10^5
48 hrs	1.5×10^2	<10	N/A	N/A
7 days	<10	<10	N/A	N/A
14 days	<10	<10	<10	<10
28 days	<10	<10	<10	<10

All results are expressed as CFU (Colony Forming Unit) per g. <= Less than N/A = Not Applicable

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Case Study 2

- Results from PE Testing at 3 month interval:

Assay date & Time	*S. aureus* AMS 027 (ATCC 6538)	*P. aeruginosa* AMS 095 (ATCC 9027)	*C. albicans* AMS 003 (ATCC10231)	*A. niger* AMS 032 (ATCC16404)	*P.Putida* AMS391 Environmental Isolate	Aeromonas hydrophila / caviae AMS392 Environmental Isolate
Inoculum CFU / mL	6.1×10^5	9.7×10^5	5.8×10^5	3.9×10^5	5.1×10^5	6.3×10^5
0 hour	3.8×10^5	7.3×10^5	5.9×10^5	3.7×10^5	2.7×10^5	6.8×10^5
48 hours	3.7×10^2	<10	N/A	N/A	<10	<10
7 days	<10	<10	N/A	N/A	<10	<10
14 days	<10	<10	<10	<10	<10	<10
28 days	<10	<10	<10	<10	<10	<10

All results are expressed as CFU (Colony Forming Unit) per g. < = less than

Case Study 2

- Product met all criteria's of PE Testing at both the initial and 3 month time point.
- Inclusion of environmental isolates demonstrated the existing preservative system (Kathon) was effective against these contaminants.
- Variables to consider: Storage conditions of product, application and transport to consumer household.

Conclusion

- Microbiological testing should be considered throughout a stability study, as a minimum at the start and end of the study.
- Parameters should be consistent with the requirements of TGO 77 – Microbiological Standards for Medicines.
- Selection of the correct pharmacopoeia and PET category is critical interpretation of results.
- Testing parameters should not be viewed as an exhaustive list.
- Environmental isolates may be considered as an addition to the pharmacopoeia PET methods.

Reference

- British Pharmacopoeia, 2012
- European Pharmacopoeia, 2012
- United States Pharmacopoeia 2012
- Therapeutic Goods Order no. 77
  Microbiological Standards for Medicines.

THANK YOU!

- ANY QUESTIONS?

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