CHALLENGE TEST

Test for evaluating the effectiveness of the preservative system in topical, nasal and ear preparations

(according to European Pharmacopoeia)

Study N°	**MMCA338/16-01**
Study Protocol code	**REL/CA0311/2016/MIC**
Sponsor	**Man Mud Inc.** **Box 872 T0L 2A0** **Turner Valley, Alberta**
Analyzed substance	**Man Mud** **Batch: 15160914A**

Summary

1 PART ONE – GENERAL INFORMATIONS

1.1 Customer

Man Mud Inc.

1.2 Tested Material

Sample	Internal code	Description
Man Mud Batch: 15160914A	CA0357/16-01	Dark green cream

1.3 *Entrusted Laboratory:*

5160 Décarie Boulevard-suite # 330
Montréal (Québec) H3X 2H9- Canada

1.4 Study Dates:

Starting date: 30/09/2016
Ending date: 04/11/2016

1.5 Laboratory Technician

Karla Castaneda

1.6 Study Director

Debora Pischedda

Note
The results reported in the present brochure refer only to the tested sample/samples and to the particular experimental conditions hereby described. This report or parts of it can be reproduced only with the experimenters' agreement.

2 PART TWO – STUDY DESIGN

2.1 Aim of the test

The Challenge Test is a predictive method useful to evaluate the effectiveness of a preserving system used in the formulation of a non-sterile cosmetic or detergent product or similar ones. By means of laboratory artificial contamination we reproduce the environmental microbial pollution of the investigated products that undergo during manufacturing storing and consumer use. This way we get important indications on the product resistance to microbial attacks and on its stability.

The manufacturing process for cosmetics and similar products does not require sterility and for this reason there is always a default level of environmental microbiological contamination that must be kept under control by a proper preservative system. Furthermore, the normal consumer use of the product causes further repeated contaminations in time.

In this assay, we overdo the experimental conditions by inoculating the samples with a very high concentration of micro-organism that is hardly found in the environment. The product is contaminated with more microbial strains, as described further on, and their reduction in growth is evaluated at different end times.

This test is conducted in accordance with that described in European Pharmacopoeia 8.0 edition in paragraph 5.1.3. (Efficacy of Antimicrobial Preservation). The test was preceded by an examination of the total microbial count of the product

2.2 Used strains and method

The inoculum is carried out with more microbial strains at different concentrations, as reported in the following table:

STRAIN	Growth Medium	Sample Inocule Concentration (CFU/g)
Pseudomonas aeruginosa ATCC 9027	Casein soya bean digest agar	2.6 X 10^6
Staphylococcus aureus ATCC 6538	Casein soya bean digest agar	2.8 X 10^6
Candida albicans ATCC 10231	Sabouraud-dextrose agar	9.9 X 10^5
Aspergillus brasiliensis ATCC 16404	Sabouraud- dextrose agar	8.7 X 10^5

The inoculum is prepared by cultivating the bacteria on casein soya bean digest agar medium and Sabouraud glucose agar for fungi, bacteria are incubated at 34 ± 1°C for 18-24h, *Candida* at 22 ± 1°C for 48h and *Aspergillus brasiliensis* at 22 ± 1°C for 5-7 days. Inoculum concentration is prepared in a physiologic solution with microbial concentration of 10^8 CFU/ml; final concentration in the tested product ranges between 10^5 and 10^6 CFU/ml.

The different microbial strains are suspended in a physiologic solution and inoculated in the tested product at a final concentration ranging between 10^5-10^6 for all the strains.

The treated samples are then stored at room temperature protected from light until plated for the microbial count. The concentration of viable cells at every end-time is determined by the plate count method, diluting the product in a solution of sodium chloride-peptone added with neutralizers of the most common preservatives (polysorbate 80, soya lecithin, thiosulfate, L-Histidine).

The microbial count at different endpoints is carried out diluting 1 g or mL of product up to $1x10^6$ times and plating each dilution in a petri dish with selective agar medium.

Plates are incubated at 34±1°C (bacteria) or at 22±1°C (yeast and mould) for the time necessary for a good growth (18-24h for bacteria, 3-7 days for yeasts and moulds). The CFU (Colony Forming Unit) value per gram or millilitre of product is obtained from the number of colonies on the plate multiplied for the dilution factor.

Plate counts are made at 2, 7, 14 and 28 days after inoculation in order to assess the dynamics of microbial reduction.

2.3 Product description and evaluation of results

The effectiveness of the preservative system is considered adequate for a preparation for topical use, according to the A criteria more restrictive, when challenge the reduction of microbial growth for at least 2 logs after 2 days, at least 3 log after 7 days for the bacteria without no subsequent increase at day 28 and at least 2 log, for fungi, after 14 days with no subsequent increase at day 28, as summarized in the following table:

		Log reduction			
		2gg	7gg	14gg	28gg
Bacteria	A	2	3	---	NI
	B	---	---	3	NI
Fungi	A	---	---	2	NI
	B	---	---	1	NI

* NI: No increase in number of viable micro-organisms compared to the previous reading.

3 PART THREE – RESULTS AND CONCLUSIONS

3.1 Results

Total microbial count

Man Mud
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STRAIN	P.aeruginosa	S.aureus	C.albicans	A.brasiliensis
CFU inoculum	$2.6x10^6$	$2.8x10^6$	$9.9x10^5$	$8.7x10^5$
CFU 2 days	<10	<10	<10	<10
Microbial reduction (log)	>5.41	>5.45	>5.00	>4.94
Reduction effectiveness (%)	>99.99	>99.99	>99.99	>99.99
CFU 7 days	<10	<10	<10	<10
Microbial reduction (log)	>5.41	>5.45	>5.00	>4.94
Reduction effectiveness (%)	>99.99	>99.99	>99.99	>99.99
CFU 14 days	<10	<10	<10	<10
Microbial reduction (log)	>5.41	>5.45	>5.00	>4.94
Reduction effectiveness (%)	>99.99	>99.99	>99.99	>99.99
CFU 28 days	<10	<10	<10	<10
Microbial reduction (log)	>5.41	>5.45	>5.00	>4.94
Reduction effectiveness (%)	>99.99	>99.99	>99.99	>99.99

Evaluation on reducing microbial growth (log)

Tempo	*Pseudomonas aeruginosa*	*Staphylococcus aureus*	*Candida albicans*	*Aspergillus brasiliensis*
2 days	> 5.41	> 5.45	> 5.00	> 4,94
7 days	> 5.41	> 5.45	> 5.00	> 4,94
14 days	> 5.41	> 5.45	> 5.00	> 4,94
28 days	> 5.41 and NI	> 5.45 and NI	> 5.00 and NI	> 4,94 and NI

3.2 Conclusions

On the bases of the following results:

STRAIN	*Does not satisfy the criteria*	*Satisfies criteria A*	*Satisfies criteria B*
Pseudomonas aeruginosa ATCC 9027		x	
Staphylococcus aureus ATCC 6538		x	
Candida albicans ATCC 10231		x	
Aspergillus brasiliensis ATCC 16404		x	

The product

Man Mud
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does not satisfy
× *satisfies according criteria A*
satisfies according to criteria B

the requirements of the preservation efficacy test for topical, nasal, ear preparations

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Prepared by
Karla Castaneda

Reviewed and approved by
Debora Pischedda

4 BIBLIOGRAPHY

1. European Pharmacopoeia, 8th Edition, General texts on microbiology- Efficacy of antimicrobial preservation. Chapter 5.1.3.
2. European Pharmacopoeia, 8th Edition, Methods of Analysis- Biological tests- Microbiological examination of non-sterile products: Microbial enumeration tests. Chapter 2.6.12.
3. European Pharmacopoeia, 8th Edition, General texts on microbiology- Microbiological quality of non-sterile pharmaceutical preparations and substances for pharmaceutical use. Chapter 5.1.4