CHALLENGE TEST

Test for evaluating the effectiveness of the preservative system in topically used products

(according to U.S.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 contaminations we reproduce the environmental microbial pollution of the investigated products, that they undergo during manufacturing, storing and consumer use. In 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 U.S.Pharmacopoeia. The test was preceded by an examination of the total microbial load 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)
Escherichia coli ATCC 8739	Casein soya bean digest agar	5.5 X 10^6
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 of fungi, bacteria are incubated at 32,5 ± 2,5°C for 18-24h, Candida at 22,5 ± 2,5°C for 44-52h and the Aspergillus at 22,5 ± 2,5°C for 6-10days

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 when 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 endpoint is carried out diluting 1 g/ml of product up to $1x10^6$ times and plating each dilution in a petri dish with selective agar medium.

The plates are kept at 32,5 ± 2,5°C (bacteria) or at 22,5 ± 2,5°C (yeast and mould) for the time necessary to a good growth (18-24h for bacteria, 3-7 days for yeasts and moulds). The U.F.C. (Unity Forming Colony) value for gram or millilitre of product is obtained from the number of colonies on the plate for the dilution factor.

To evaluate the microbial reduction in time, plate counts are carried out at four end-times normally after 24 or 48 hours, 7, 14 and 28 days from the starting inoculum.

2.3 *Product description and evaluation of results*

For the purpose of testing, the samples have been divided into four categories. The criteria of antimicrobial effectiveness for these products are a function of the route of administration.

Category	Product description	Antimicrobial effectiveness from	
		Bacteria	Yeast and Molds
1	Injections, other parenterals including emulsions, otic products, sterile nasal products, and ophthalmic products made with aqueous bases or vehicles	NTL 1.0 log reduction from the initial calculated count at 7 days, NTL 3.0 log reduction at 14 days No increase from the 14days count at 28 days	No increase from the initial calculated count at 7, 14 and 28 days
2	Topically used products made with aqueous bases or vehicles, non-sterile nasal products, and emulsion, including those applied to mucous membranes	NTL 2.0 log reduction from the initial count at 14 days, and no increase from the 14 days count at 28 days	No increase* from the initial calculated count at 14 and 28 days
3	Oral products other than antacids, made with aqueous bases or vehicles	NTL 1 log reduction at 14 days No increase at 28 days	No increase from the initial calculated count at 14 and 28 days
4	Antacids made with an aqueous base	No increase from the initial calculated count at 14 and 28 days	No increase from the initial calculated count at 14 and 28 days

*not more than 0.5log10 unit

3 PART THREE – RESULTS AND CONCLUSIONS

3.1 Results

Total microbial count

Man Mud
Batch: 15160914A

STRAIN	E.coli	P.aeruginosa	S.aureus	C.albicans	A.brasiliensis
CFU inoculum	$5.5x10^6$	$2.6x10^6$	$2.8x10^6$	$9.9x10^5$	$8.7x10^5$
CFU 2 days	<10	<10	<10	<10	<10
Microbial reduction (log)	>5.74	>5.41	>5.45	>5.00	>4.94
Reduction effectiveness (%)	>99.99	>99.99	>99.99	>99.99	>99.99
CFU 7 days	<10	<10	<10	<10	<10
Microbial reduction (log)	>5.74	>5.41	>5.45	>5.00	>4.94
Reduction effectiveness (%)	>99.99	>99.99	>99.99	>99.99	>99.99
CFU 14 days	<10	<10	<10	<10	<10
Microbial reduction (log)	>5.74	>5.41	>5.45	>5.00	>4.94
Reduction effectiveness (%)	>99.99	>99.99	>99.99	>99.99	>99.99
CFU 28 days	<10	<10	<10	<10	<10
Microbial reduction (log)	>5.74	>5.41	>5.45	>5.00	>4.94
Reduction effectiveness (%)	>99.99	>99.99	>99.99	>99.99	>99.99

Evaluation on reducing microbial growth

Product category: 2

Time	*Escherichia coli*	*Pseudomonas aeruginosa*	*Staphylococcus aureus*	*Candida albicans*	*Aspergillus brasiliensis*
14 days	Effective (> 2log)	Effective (> 2log)	Effective (> 2log)	Effective (no increase*)	Effective (no increase*)
28 days	Effective (no increase*)	Effective (no increase*)	Effective (no increase*)	Effective (no increase*)	Effective (no increase*)

*not more than 0.5log10 unit

3.2 Conclusions

On the bases of the following results here shown,

STRAIN	Does not satisfies the criteria	Satisfies criteria
Escherichia coli ATCC 8739		x
Pseudomonas aeruginosa ATCC 9027		x
Staphylococcus aureus ATCC 6538		x
Candida Albicans ATCC 10231		x
Aspergillus brasiliensis ATCC 16404		x

The product **Man Mud - Batch: 15160914A**, satisfies the requirements of the preservation efficacy test for topically used products according to USP regulation.

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