KISTEC29-114B02

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MESSRS: [redacted]

MESSRS: SBM plus Co. Ltd

([redacted])

Determination of antiviral activity using influenza virus

Kanagawa Institute of Industrial Science and Technology(KISTEC)
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Authorizer signature

Researcher

M. Hashimoto

1. Test conditions

a) Reference to this test method : the reference sheet No.2

b) Characterization of the sample :

- Type : liquid
- Sample name: [redacted] ECO PURE Keep Clean Powder
- Control sample name : PBS

c) Pre-exposure to UV light : non

d) Disinfection of specimen : non

e) Type of test strains number :

- Influenza A virus (H1N1) A/PR/8/34 strain (ATCC VR-1469)
- Host cell: MDCK cell (ATCC CCL-34)

f) Light source : White Fluorescent lamp (FL20SSW/18, MITSUBISHI)

g) Illuminance meter : IM-600M (Topcon)

h) Light exposure conditions :

- Intensity: 500 lx
- Duration: 4h
- UV sharp cut filter: Condition B (under 380 nm cut-off condition)

i) Type and size of moisture preservation glass

- Moisture preservation glass: Borosilicate glass (100mm × 100mm)

j) Test date : Jan. 17th, 2018

k) Virus titer determination: $TCID_{50}$ method by the luminescence using the Viral ToxGlo Assay ™ kit (Promega)

2. Result

Influenza virus	4h 500 lx Viral concentration ($TCID_{50}$/test mixture ml)*1
PBS	1.7E+05 ($B_{B\text{-}500}$)
ecolala CLEAN ECO PURE	2.2E+03 ($C_{B\text{-}500}$)

*1 "E+05" represents "$\times 10^5$".

- **$V_{B\text{-}500}$:Antiviral activity = 1.9**

Antivirall activity (with light irradiation) : $[V_{F\text{-}L}=\mathrm{Log}(B_{F\text{-}L})-\mathrm{Log}(C_{F\text{-}L})]$

F:type of UV cut-off condition, L:illuminance of indoor light

B: viral concentration of control sample, C: viral concentration of sample

- Viral concentration of initial test mixture : 7.8E+05 $TCID_{50}$/ml
- Detection limit from cytotoxic test of the sample : 2.0E+03 $TCID_{50}$/ml
- Sample (5 ml) and viral suspension (0.15 ml) were mixed in ϕ60 mm dish and irradiated.

Test method for antiviral test (influenza rivus)

①Prepararation of virus stock (Fig. 1)

Influenza virus (Influenza A virus (H1N1) A / PR / 8/34 strain) is infected with MDCK (Madin-Darby canine kidney) cells which are host cells (a). After cultivation, when the cytopathic effect (CPE) of more than 90% is observed , the culture supernatant is collected (b) and purified by filtering and centrifugation, which is stocked virus suspension.

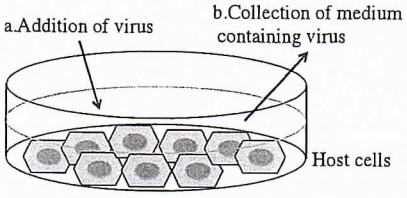

Fig. 1 Preparation of virus

②Cytotoxicity test

Components coated on the test sample may be eluted during the test and recovery steps, and thus it is concerned to damage the host cell or inhibit the infection of virus. If such a cytotoxic sample is tested, it is not possible to accurately evaluate antiviral activity. Therefore, before this test, toxicity test is recommended to confirm the toxicity of the test sample itself. In the absence of toxicity, we will perform this test. If cytotoxicity is observed, we consider the strength of toxicity and judge the applicability of this test.

③Test method (Fig. 2 and 3)

At first, virus stock suspension as prepared in ① is diluted with phosphate buffer (PBS) to an appropriate concentration (10 times or more) and use it as the test virus suspension. Next, 150 µl of this test virus suspension (change as needed to 75-400 µl) is inocculated on the blank and test samples, and then cover by cover film (Fig. 2). These samples are laid under the dark or light condition (UV light or visible light, using sharp cut filter as necessary, Fig. 3) at 25 ° C ± 3 ° C in the humidity environment. Irradiation time is standardized to 4 hours. After the predetermined period of time, the test virus suspension is collected with 5 ml of PBS. "0 hour" means to collect the virus immediately after inoculation.

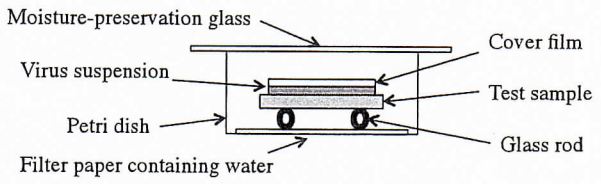

Fig. 2 construction of test

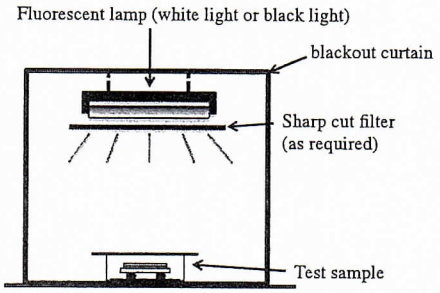

Fig. 3 irradiation apparatus

④Determination of virus titer (Fig. 4)

The virus suspension collected in ③ is serially diluted at 10-fold using with PBS and then add 50 µl of thses diluted suspension to MDCK cells which were previously cultured on a 96-well plate. These cells are cultivated for 4 to 5 days at 34 ° C and 5% CO_2 condidion. After incubation, virus titer is determined by $TCID_{50}$ method (50% Tissue Culture Infectious Dose: the concentration of the virus when half of the host cells are infected with the virus) using the luminescence method using the Viral ToxGlo Assay ™ kit (Promega).

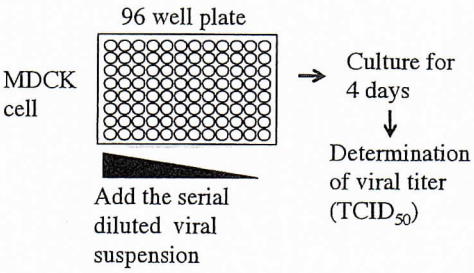

Fig. 4 determination of viral titer

⑤Calculation of antiviral actibity

Antiviral activity is calculated using each values of $TCID_{50}$ by following formation.

Antiviral activity under light condition (V_L) = $\log(B_L) - \log(C_L)$

Antiviral activity under dark condition (V_D) = $\log(B_D) - \log(C_D)$

Test result ($TCID_{50}$/sample)

influenza virus	0 hr	△ hr	
		dark	light
blank	A	B_D	B_L or $B_{F\text{-}L}$
test sample		C_D	C_L or $C_{F\text{-}L}$

L:intensity of UV

F-L:type of the sharp cut filter and intensity of visible light

KISTEC29-114A01

Issue date : Nov. 27th, 2017

[redacted]

MESSRS: SBM plus Co. Ltd

[redacted]

Determination of antibacterial activity

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Authorizer signature

Researcher

N. Igarashi

1. Test conditions

a) Reference to this test method :ISO 17094: 2014 (Antibacterial activity of indoor-light-sensitive photocatalyst) and EN 1040: 2005 (Disinfectant)

b) Characterization of the photocatalytic and non-treated samples :

- Type : liquid
- Photocatalytic sample name : [redacted]ECO PURE/Keep Clean Powder
- Non-treated sample name : PBS (Control)

c) Pre-exposure to UV light : No exposure

d) Disinfection of sample : No disinfection

e) Type of test strains number :

- *Escherichia coli* (NBRC 3972)
- *Staphylococcus aureus* (NBRC 12732)

f) Light source : White Fluorescent lamp (FL20SSW/18, MITSUBISHI)

g) Illuminance meter : IM-600M (Topcon)

h) Light exposure conditions :

- Intensity: 500 lx
- Duration: 0, 30, 60, 240 min
- UV sharp cut filter: Condition B (under 380 nm cut-off condition)

i) Moisture preservation glass : Borosilicate glass

j) Test date : Nov. 22nd, 2017

2. Result

E. coli	Number of viable bacteria (cfu/test mixture ml)*1				R_{B-500}:Antibacterial activity (With light irradiation)*2		
	0 min	500 lx			500 lx		
		30 min	60 min	240 min	30 min	60 min	240 min
PBS (Control)	1.7E+07	1.5E+07	1.4E+07	9.7E+06	-	-	-
ecolala CLEAN/ ECO PURE/ Keep Clean Powder	-	<1.0E+01	1.0E+01	<1.0E+01	6.1	6.1	5.9

*1 "E+07" represents "$\times 10^7$".

*2 Referential value due to being non-compliant with JIS R 1752 (ISO 17094) test method.

Number of viable bacteria in test suspension :1.6E+08 cfu/ml (suspended in 1/500 NB)

Test mixture of sample (4 ml), bacterial suspension (0.5ml) and sterilized water (0.5ml) was irradiated.

Antibacterial activity (with light irradiation) : $[R_{F\text{-}I}=\mathrm{Log}(U_{F\text{-}I})-\mathrm{Log}(T_{F\text{-}I})]$

F:type of UV cut-off condition, I:illuminance of indoor light

U: number of viable bacteria of non-treated sample,

T: number of viable bacteria of photocatalytic treated sample

ISO 17094:2014

Fine ceramics (advanced ceramics, advanced technical ceramics) – Test method for antibacterial activity of semiconducting photocatalytic materials under indoor lighting environment

Antibacterial activity value calculation

Indoor-light-active photocatalyst antibacterial value

$R_{F\text{-}I} = [\log_{10}(B_{F\text{-}I}/A) - \log_{10}(C_{F\text{-}I}/A)] = \log_{10}(B_{F\text{-}I}/C_{F\text{-}I})$

$\Delta R = \log_{10}[B_{F\text{-}I}/C_{F\text{-}I}] - [\log_{10}(B_D/A) - \log_{10}(C_D/A)] = \log_{10}[B_{F\text{-}I}/C_{F\text{-}I}] - \log_{10}[B_D/C_D]$

where

$R_{F\text{-}I}$ is the indoor-light-active photocatalyst antibacterial activity value, after indoor light illumination of intensity (F-I);

F is the type of UV cut-off condition (condition A or condition B);

I is the indoor light illumination intensity (lx);

A is the average number of viable bacteria of non-treated test pieces, just after inoculation (cfu);

$B_{F\text{-}I}$ is the average number of viable bacteria of non-treated test pieces, after indoor light illumination of intensity (F-I) (cfu);

$C_{F\text{-}I}$ is the average number of viable bacteria of indoor-light-active photocatalyst treated test pieces, after indoor light illumination of intensity (F-I) (cfu);

ΔR is the indoor-light-active photocatalyst antibacterial activity value with indoor light illumination;

B_D is the average number of viable bacteria of non-treated test pieces, after being kept in a dark place (cfu);

C_D is the average number of viable bacteria of indoor-light-active photocatalyst treated test pieces, after being kept in a dark place (cfu).

JFRL

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http://www.jfrl.or.jp/

No. 17120860001-0201 1/1
Date issued: November 02, 2017

CERTIFICATE OF ANALYSIS

Client: [redacted]
[redacted]
SBMplus co., Ltd.
1-3-6 Higashikanda Chiyoda-ku Tokyo 101-0031 JAPAN

Sample name: [redacted]
ECOPURE

Received date: October 20, 2017

This is to certify that the following result(s) have been obtained from our analysis on the above-mentioned sample(s) submitted by the client.

Test Result(s)

Test Item	Result	QL	N	M
Coal-tar dyes approved by Japanese Food Additives Standards	Negative	.....		1
Coal-tar dyes not approved by Japanese Food Additives Standards	Negative	.....		1
Methanol	Not detected	20 ppm		2
Arsenic (as As)	Not detected	0.1 ppm		3
Heavy metals (as Pb)	Not detected	5 ppm		4
Lead	Not detected	0.05 ppm		3
Cadmium	Not detected	0.01 ppm		3
Mercury	Not detected	0.01 ppm		5

QL: Quantitation limit N: Notes M: Method

Method
1:Thin layer chromatography
2:Gas chromatography
3:Atomic absorption spectrometry
4:Sodium sulfide colorimetric method
5:Cold vapor atomic absorption spectrometry

JAPAN FOOD RESEARCH LABORATORIES

Signed for and on behalf of JFRL

T. Arai — Nov. 02, 2017

Takeko Arai — Date
Section of Analysis Documentation

Japan Food Research Laboratories
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http://www.jfrl.or.jp/

Date issued: November 23, 2017

REPORT

Client: [redacted]
[redacted]
SBMplus co., Ltd
1-3-6 Higashikanda Chiyoda-ku Tokyo 101-0031 JAPAN

Test sample(s): [redacted]
ECOPURE

Title: Acute Oral Toxicity Test in Female Mice

Received date of test sample(s): October 05, 2017

This report has been translated into English from Japanese report No. 17113741001-0101 (Date issued: November 23, 2017).

Signed for and on behalf of JFRL

JAPAN FOOD RESEARCH LABORATORIES

T. Arai
Takeko Arai
Section of Analysis Documentation

Dec. 14, 2017
Date

Acute Oral Toxicity Test in Female Mice

Abstract

The test sample was tested for acute oral toxicity in female mice. The test sample was orally administered to animals at a single dose of 5000 mg/kg b.w. (body weight), and they were observed for 14 days. As a result, the test sample caused no death in any of the mice during the observation period. Consequently, the LD50 value (single dose, oral administration) of the test sample is considered to be more than 5000 mg/kg b.w. in female mice.

1. Client

Global Ecology co., Ltd
SBMplus co., Ltd

2. Test sample

ecolala CLEAN
ECOPURE

3. Test facility

Tama Laboratory, Japan Food Research Laboratories
6-11-10 Nagayama, Tama-shi, Tokyo 206-0025, Japan

4. Test period

From October 05, 2017 to November 23, 2017

5. Purpose

The acute oral toxicity in female mice of the test sample is evaluated according to OECD Guideline for Testing of Chemicals 420 (2001).

6. Preparation of test dilution

The test sample was diluted with water for injection to make 250 mg/mL test dilution.

7. Experimental animals

Female mice of ICR strain, at an age of 5 weeks, were purchased from Japan SLC, Inc. Before test, they were acclimated to laboratory conditions for about 1 week to verify that no abnormalities were shown in general conditions. They were housed in plastic cages (five animals per cage) under standard laboratory conditions (Temperature: 23 ˚C ± 3 ˚C, Light-dark cycle: 12/12 hours). Feed (Labo MR Stock diet, Nosan Corporation) and tap water were provided *ad libitum* throughout the experiment.

8. Procedures

Female mice were allocated into experimental and control groups each consisting of five mice.

The mice were not fed for about 4 hours before administration. After measurement of body weight, the animals in the experimental group were orally administered with the test dilution at a single dose of 20 mL/kg b.w. (at a dosage of 5000 mg/kg b.w. test sample) using a stomach tube. The animals in the control group were administered with water for injection, as vehicle control, at the same dose.

The clinical observation was carried out frequently on the day of the administration and once a day for the following 13 days. The body weight was measured after 7 and 14 days of the administration. The mean body weight values of the experimental group and the control group were assessed for homogeneity of variance by Levene's test. Since the Levene's test was not significant, Student's t-test was applied for the comparison of two groups ($\alpha = 0.05$).

At the completion of the test, all of the mice were sacrificed for necropsy.

9. Results

1) Death of animals

None of the mice died during the experimental period.

2) Clinical observations

No abnormalities were observed in any of the mice during the experimental period.

3) Body-weight changes (Table 1)

After 14 days of administration, one mouse in the experimental group exhibited a weight loss.

After 7 days of administration, no significant difference in body weight was detected between the experimental group and the control group. After 14 days, however, the body weight of the animals in the experimental group was significantly lower ($p<0.05$) than that in the control group.

4) Necropsy

No remarkable changes were found in any of the mice.

10. Conclusion

The acute oral toxicity in female mice of the test sample was determined.

Oral administration of 5000 mg/kg b.w. test sample caused no death in any of the mice during the observation period.

Consequently, the LD50 value (single dose, oral administration) of the test sample is considered to be more than 5000 mg/kg b.w. in female mice.

Table 1. Body-weight changes

Group	Body weight (Units: g)		
	Pre-administration	7 days	14 days
Experimental group	28.4 ± 1.1 (5)	30.3 ± 1.8 (5)	31.0 ± 1.7* (5)
Control group	28.5 ± 1.2 (5)	31.7 ± 1.2 (5)	34.0 ± 0.7 (5)

The values are mean ± SD.

The values in parentheses represent the number of animals.

* A significant difference is detected between the experimental and control groups ($p<0.05$).

End of Report