

 BioPharma Product Testing	Test Facility Eurofins Biolab S.r.l.	Report N°: S-2016-02257 SAM Version: English Page: 5 of 7 Print date: July 25 th , 2016
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Experimental Report S-2016-02257– Antimicrobial Efficacy

EXPERIMENTAL PROCEDURE

ASSAY SYSTEM

Identification of the Test microorganisms

Cultures of the following microorganism were used:

- *Staphylococcus aureus* MRSA ATCC 33592
- *Salmonella typhimurium* ATCC 13311
- *Escherichia coli* ATCC 8739
- *Streptococcus uberis* ATCC 19436
- *Clostridium perfringens* ATCC 13124
- *Enterococcus faecium* ATCC 6057
- *Pseudomonas aeruginosa* ATCC 9027
- *Klebsiella pneumoniae* ATCC 10031
- *Candida albicans* ATCC 10231
- *Trichophyton verrucosum* ATCC 28943

CULTURAL MEDIA AND REAGENTS

The validity of cultural media and reagents were checked before starting the analyses.

Saline solution

Neutralizer CEN

Tryptone Soya Agar (TSA)

Sabouraud Dextrose Agar (SDA)

Potato dextrose agar (PDA)

Schaedler blood Agar

Yeast extract

EQUIPMENT AND GLASSWARE

The validity of instruments and equipment were checked before starting the analyses.

Ordinary microbiology laboratory equipment and in particular:

Thermostat @ 32.5°±2.5°C

Thermostat @ 22.5°±2.5°C

Thermostat @ 30°±1°C

Jars for anaerobic condition

EXPERIMENTAL DESIGN

Preparation of microbial suspension

Microorganism suspensions were prepared with a concentration from 10⁷ to 10⁸ cfu/ml.

The number of cfu/ml of each microbial suspension was verified by pour plate method.

Preparation of the assay sample

The assay sample was prepared by mixing 4g of test item into 5 ml of NaCl solution 0.9% in sterile tubes, one aliquot was prepared for each test strain.

Preparation of the negative control

NaCl solution 0.9% was used neat and aliquots of 9-mL of in sterile tubes were prepared in sterile tubes, one aliquot was prepared for each test strain.

Contact times

The counts were performed after the following contact times: 2 hours and 24 hours.

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Incubation Conditions

Bacteria: in TSA at 30-35°C under aerobic condition for 5 days.

Clostridium perfringens ATCC 13124 in Schaedler blood Agar at 32.5°±2.5°C under anaerobic condition for 5 days.

Enterococcus faecium ATCC 6057: in Schaedler blood Agar at 32.5°±2.5°C under aerobic condition for 5 days.

Trichophyton verrucosum ATCC 28943 in PDA + 0.5% Yeast extract at 30°±1°C for 7 days.

EXECUTION OF THE ASSAY

Contamination control test

Initial plate count test on original uninoculated sample was performed. This test was only carried at t₀. The assay sample was diluted 1: 10 with neutralizer and mixed well. Counts in duplicate by pour plates in TSA for bacteria and SDA for mold were prepared and the appropriate incubation conditions were adopted.

Inoculation of the assay samples

For each test strain an aliquot of the assay sample and negative control was prepared into sterile containers, then each aliquot was inoculated separately with 1 ml of the test suspension, in order to obtain a final concentration of 1x10⁵ - 1x10⁶ cfu/ml for bacteria and mold.

After each inoculation the vials were mixed in order to make inoculum homogeneously dispersed.

The inoculated test material samples were kept at 20-25°C under aerobic/anaerobic condition depending on the specific demands of each organism for the whole duration of the assay.

Recovery of Inoculated Test Microorganisms

At t₀ time and at each appointed contact times, 1 ml of the inoculated material was transferred into a tube neutralizer and was vortex stirred.

Serially dilutions 1:10, down to 10⁻⁵ in neutralizer were prepared and each dilution were performed in duplicate by inclusion or spread method into appropriate agar medium depending on the specific demands of each organism.

Neutralization Verification

For each test strain, 1 ml of the assay sample, was transferred respectively into a tube containing 9 ml (dilution 1:10) of Diluent, vortex stirred and left for 5 minutes, then inoculated separately with 1 ml of test suspension at 10³ cfu/ml of each test strain. Counts were performed in duplicate by inclusion or spread method into appropriate agar medium depending on the specific demands of each organism.

PROCESSING OF THE RESULTS

Viability reduction of microorganism at the end of the contact time is expressed as % according to the following equation:

$$R(\%) = \frac{(N - N_{ix})}{N} \times 100$$

Where:

N: is the average of the number of viable bacteria in inoculum (cfu/ml).

N_{ix}: is the average of the number of viable bacteria, recovered at each different contact time.

ASSAY VALIDITY CRITERIA

The test is valid if in Neutralization Verification, for each test strain, the number of viable microorganisms recovered in the presence of the product, does not differ by a factor greater than 2 (recovery between 50-200%) from the number of viable microorganisms recovered in the control in absence of product.

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RESULTS

Results are reported in Addendum #1.

The microbial counting values in the validation of the neutralization are shown Table N. 1.

The microbial counting values for the concentration of test suspensions and the viable count at the different contact time Table N. 2.

The percentage reductions in the number of microorganisms at the different contact times are shown in Table N. 3.

DEVIATIONS

No deviation has been recorded.

CONCLUSIONS

On the basis of the results recorded, the test material "Nordic STA Hyg", **has antimicrobial** activity as it causes a reduction of 100% after 2h and 24 h of contact time for bacteria and yeast, a reduction of 99.83% after 2h and 100% after 24h of contact time for *Trichophyton verrucosum*.

ADDENDUM

ADDENDUM	TITLE	PAGE NUMBER
#1	ANTIMICROBIAL EFFICACY	2

Eurofins Biolab S.r.l.

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Study n.º: S-2016-02257 SAM

Sample: Nordic STA Hyg

Batch: 6/6-16

Started on: 13/07/16

Table n.1: NEUTRALIZER VERIFICATION

Inoculum	<i>Escherichia coli</i> ATCC 8739	<i>Klebsiella pneumoniae</i> ATCC 10031	<i>Salmonella typhimurium</i> ATCC 13311	<i>Enterococcus faecium</i> ATCC 6057	<i>Staphylococcus aureus</i> MRSA ATCC 33592	<i>Streptococcus uberis</i> ATCC 19436	<i>Clostridium perfringens</i> ATCC 13124	<i>Pseudomonas aeruginosa</i> ATCC 9027	<i>Candida albicans</i> ATCC 10231	<i>Trichophyton verrucosum</i> ATCC 28943
in neutralizer	200	280	275	200	210	180	220	190	260	48
in product	160	230	250	150	136	145	160	120	241	43
Recovery %	80.00	82.14	90.91	75.00	64.76	80.56	72.73	63.16	92.69	89.58

Table n.2: MICROBIAL COUNTING VALUES (cfu/ml)

Test suspension (cfu/ml)	<i>Escherichia coli</i> ATCC 8739	<i>Klebsiella pneumoniae</i> ATCC 10031	<i>Salmonella typhimurium</i> ATCC 13311	<i>Enterococcus faecium</i> ATCC 6057	<i>Staphylococcus aureus</i> MRSA ATCC 33592	<i>Streptococcus uberis</i> ATCC 19436	<i>Clostridium perfringens</i> ATCC 13124	<i>Pseudomonas aeruginosa</i> ATCC 9027	<i>Candida albicans</i> ATCC 10231	<i>Trichophyton verrucosum</i> ATCC 28943
	2.2E+07	3.0E+07	3.2E+07	1.2E+07	2.1E+07	2.4E+07	2.0E+07	1.3E+07	3.2E+07	4.0E+07

Contact time	<i>Escherichia coli</i> ATCC 8739	<i>Klebsiella pneumoniae</i> ATCC 10031	<i>Salmonella typhimurium</i> ATCC 13311	<i>Enterococcus faecium</i> ATCC 6057	<i>Staphylococcus aureus</i> MRSA ATCC 33592	<i>Streptococcus uberis</i> ATCC 19436	<i>Clostridium perfringens</i> ATCC 13124	<i>Pseudomonas aeruginosa</i> ATCC 9027	<i>Candida albicans</i> ATCC 10231	<i>Trichophyton verrucosum</i> ATCC 28943
T0	< 1.0E+01	1.2E+05	1.0E+05	< 1.0E+01	< 1.0E+01	< 1.0E+01	< 1.0E+01	< 1.0E+01	3.0E+05	5.0E+05
2 hour	< 1.0E+01	< 1.0E+01	< 1.0E+01	< 1.0E+01	< 1.0E+01	< 1.0E+01	< 1.0E+01	< 1.0E+01	1.1E+02	7.0E+04
24 hours	< 1.0E+01	< 1.0E+01	< 1.0E+01	< 1.0E+01	< 1.0E+01	< 1.0E+01	< 1.0E+01	< 1.0E+01	< 1.0E+01	< 1.0E+01

Table n.3: % REDUCTIONS OF VIABLE COUNT

Contact time	<i>Escherichia coli</i> ATCC 8739	<i>Klebsiella pneumoniae</i> ATCC 10031	<i>Salmonella typhimurium</i> ATCC 13311	<i>Enterococcus faecium</i> ATCC 6057	<i>Staphylococcus aureus</i> MRSA ATCC 33592	<i>Streptococcus uberis</i> ATCC 19436	<i>Clostridium perfringens</i> ATCC 13124	<i>Pseudomonas aeruginosa</i> ATCC 9027	<i>Candida albicans</i> ATCC 10231	<i>Trichophyton verrucosum</i> ATCC 28943
T0	100.00	99.60	99.69	100.00	100.00	100.00	100.00	100.00	99.06	98.75
2 hour	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	99.83
24 hours	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

Study n. #: S-2016-02257 SAM

Sample: NaCl (negative control)

Started on: 13/07/16

Table n.1: NEUTRALIZER VERIFICATION

Inoculum	<i>Escherichia coli</i> ATCC 8739	<i>Klebsiella pneumoniae</i> ATCC 10031	<i>Salmonella typhimurium</i> ATCC 13311	<i>Enterococcus faecium</i> ATCC 6057	<i>Staphylococcus aureus</i> MRSA ATCC 33592	<i>Streptococcus uberis</i> ATCC 19436	<i>Clostridium perfringens</i> ATCC 13124	<i>Pseudomonas aeruginosa</i> ATCC 9027	<i>Candida albicans</i> ATCC 10231	<i>Trichophyton verrucosum</i> ATCC 28943
in neutralizer	200	280	275	200	210	180	220	190	260	48
in product	180	250	248	160	190	160	200	170	230	43
Recovery %	90.00	89.29	90.18	80.00	90.48	88.89	90.91	89.47	88.46	89.58

Table n.2: MICROBIAL COUNTING VALUES (cfu/ml)

Test suspension (cfu/ml)	<i>Escherichia coli</i> ATCC 8739	<i>Klebsiella pneumoniae</i> ATCC 10031	<i>Salmonella typhimurium</i> ATCC 13311	<i>Enterococcus faecium</i> ATCC 6057	<i>Staphylococcus aureus</i> MRSA ATCC 33592	<i>Streptococcus uberis</i> ATCC 19436	<i>Clostridium perfringens</i> ATCC 13124	<i>Pseudomonas aeruginosa</i> ATCC 9027	<i>Candida albicans</i> ATCC 10231	<i>Trichophyton verrucosum</i> ATCC 28943
	2.2E+07	3.0E+07	3.2E+07	1.2E+07	2.1E+07	2.4E+07	2.0E+07	1.3E+07	3.2E+07	4.0E+07

Contact time	<i>Escherichia coli</i> ATCC 8739	<i>Klebsiella pneumoniae</i> ATCC 10031	<i>Salmonella typhimurium</i> ATCC 13311	<i>Enterococcus faecium</i> ATCC 6057	<i>Staphylococcus aureus</i> MRSA ATCC 33592	<i>Streptococcus uberis</i> ATCC 19436	<i>Clostridium perfringens</i> ATCC 13124	<i>Pseudomonas aeruginosa</i> ATCC 9027	<i>Candida albicans</i> ATCC 10231	<i>Trichophyton verrucosum</i> ATCC 28943
T0	1.4E+05	2.0E+05	2.4E+05	1.0E+05	1.8E+05	1.3E+05	1.7E+05	1.0E+05	2.7E+05	1.9E+05
2 hour	7.5E+04	1.5E+05	2.0E+05	7.8E+04	1.3E+05	1.2E+05	1.3E+04	1.2E+05	2.5E+05	5.0E+04
24 hours	7.0E+04	1.6E+05	2.2E+05	5.6E+04	1.1E+05	1.4E+05	1.0E+04	1.0E+05	2.0E+05	3.0E+05

Table n.3: % REDUCTIONS OF VIABLE COUNT

Contact time	<i>Escherichia coli</i> ATCC 8739	<i>Klebsiella pneumoniae</i> ATCC 10031	<i>Salmonella typhimurium</i> ATCC 13311	<i>Enterococcus faecium</i> ATCC 6057	<i>Staphylococcus aureus</i> MRSA ATCC 33592	<i>Streptococcus uberis</i> ATCC 19436	<i>Clostridium perfringens</i> ATCC 13124	<i>Pseudomonas aeruginosa</i> ATCC 9027	<i>Candida albicans</i> ATCC 10231	<i>Trichophyton verrucosum</i> ATCC 28943
T0	99.36	99.33	99.25	99.17	99.14	99.46	99.15	99.23	99.16	99.53
2 hour	99.66	99.50	99.38	99.35	99.38	99.50	99.94	99.08	99.22	99.88
24 hours	99.68	99.47	99.31	99.53	99.48	99.42	99.95	99.23	99.38	99.25