VIRIDIS BioPharma

Report prepared for
American Biotech Laboratory

ANTIVIRAL ACTIVITY OF ASAP

March 2003- June 2003

By:
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*ASAP Nano-Silver now known as Argasol™ - Powered by SilverSol® Technology

Appendix A

Sample Source : Amreican Biotech Laboratories

Sample Identification : ASAP 22 (Lot # 02193)

Date of Analysis : 29th October 2002

Report issue date : 26th November 2002

Total No. of pages : Two

Antiviral Activity of ASAP Solution

Aim : To check antiviral activity of ASAP Solution (22 ppm) using a bacteriophage model.

Culture Used : T – even phage
E.coli host

Method :

Phage virulence

Virulence of Phage was activated by performing 3 successive transfers in the host and extracting with chloroform. The virulence of the Phage was checked by spotting on *E.coli* (host) lawn and checking for zone of lysis.

Experimental conditions

10 ml of ASAP solution was challenged with 1 ml of Phage suspension (10^9 Phage particles). Similarly a negative control was run using 10 ml saline in lieu of ASAP solution. 20 µl aliquots were withdrawn from 0 hour onwards at 30 minutes intervals and presence of Phage was determined using the host indicator system. Results are as per Table-1.

TABLE-1:

Sr. No.	Exposure Time (Hrs.)	Test		Control	
		Zone of Lysis	Presence of Phage	Zone of Lysis	Presence of Phage
1.	0	+++	Present	+++	Present
2.	0.5	+++	Present	+++	Present
3.	1	++	Present	+++	Present
4.	1.5	+	Present	+++	Present
5.	2	2 viral particles	Present	+++	Present
6.	2.5	-	Absent	+++	Present

Conclusion : The ASAP solution showed virucidal activity completely eliminating all viral particles in a period of 2.5 hours. The negative control samples showed presence of Phage after 2.5 hours under similar conditions. Though silver is postulated to exert anti-microbial activity by uncoupling the ETC mechanism in prokaryotes, it is acting through a different mechanism in this case possibly through precipitation of the viral proteins. These results could be extrapolated and it would be interesting to determine antiviral activity against known pathogenic animal viruses using a tissue culture model.

KILL-TIME STUDIES
SPORICIDAL ACTIVITY OF
ASAP SILVER SOLUTION

(Submitted January 30, 2003)

February 10, 2003

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I. PURPOSE.

The purpose of these studies was to determine the antimicrobial activity of two silver-based disinfectants on bacterial endospores from the test organism *Bacillus subtilis*. This was accomplished by performing standard kill-time assays using a suspension of *B. subtilis* endospores.

II. MATERIALS AND METHODS.

A. Test organism.

A test suspension containing endospores from *Bacillus subtilis* (ATCC # 19659) was prepared from a culture grown on Nutrient Agar, to which additional sporulation enhancements were added. Plates were harvested with sterile water and endospores were purified by repeated centrifugations and resuspensions in water. The final wash was in 70% ethanol for 30 min, to ensure the death of all vegetative bacteria. The spores were re-suspended in water containing 0.1% Tween 80 to prevent clumping.

B. Neutralizer.

The Neutralizer mixture consisted of 12.7% Tween 80, 6.0% Tamol, 1.7% lecithin, 1% Peptone, and 0.1% Cystine.

C. Kill-Time Procedure.

1. A 9.9 ml aliquot of the disinfectants (American Biotech Labs experimental solutions: one containing 14 ppm silver and 1.5% H_2O_2; the other containing 10 ppm silver and 1.0% H_2O_2) was placed in a sterile 20 mm x 150 mm tube. The tube was equilibrated in a 20°C water bath.
2. The tube of disinfectant was inoculated with 100 µl of the test organism suspension at time zero.
3. At 10 min, 30 min, 1 hr, 2 hr, 4 hr, 6 hr, and 8 hr, one ml of organism/disinfectant suspension was removed to nine ml of neutralizer. The tube was mixed thoroughly.
4. After two min, the neutralized suspension was serially diluted 1:10, in physiological saline solution (PSS).
5. The number of viable organisms in selected dilution tubes was assayed by membrane filtration. One ml aliquots were plated in duplicate. The membranes were washed with about 100 ml of sterile PSS and removed to Columbia Agar plates. The plates were incubated at 37°C for 20 hr.
6. The number of colonies on each filter was counted and log reductions computed.

D. Controls.

1. Titers of the test suspensions were computed by performing membrane filtration assays of selected 1:10 dilutions of the test suspensions in PSS.
2. A neutralizer control was performed by inoculating a mixture of 9 ml of neutralizer and 1 ml of disinfectant with 100 µl of the $1:10^3$ dilution of the titer. This produced about 2,000 CFU / ml in the tube, which was allowed to stand for 20 minutes prior to diluting 1:10. Both tubes were assayed by membrane filtration using duplicate 1 ml samples.

III. RESULTS.

***Bacillus subtilis* Spores:**

Titer.

	Dilution:		
	$1:1x10^6$	$1:1x10^7$	$1:1x10^8$
Number of colonies:	TNC	36	5
	TNC	27	4

Solution containing 14 ppm silver and 1.5% H_2O_2:

Dilution of *B. subtilis* spore/disinfectant suspension:

Time	$1:1x10^1$	$1:1x10^2$	$1:1x10^3$	$1:1x10^4$	$1:1x10^5$
10 min	-	-	TNC	TNC	227
	-	-	TNC	TNC	265
30 min	-	-	TNC	TNC	258
	-	-	TNC	TNC	273
1 hr	-	-	TNC	TNC	55
	-	-	TNC	TNC	33
2 hr	-	TNC	207	29	-
	-	TNC	237	24	-
4 hr	59	3	1		
	57	5	1		
6 hr	0	0	0		
	3	0	0		
8 hr	1	0	0		
	1	0	0		

Neutralization Control.

Undiluted	$1:1x10^1$
TNC	195
TNC	210

Solution containing 10 ppm silver and 1.0% H_2O_2:

Dilution of *B. subtilis* spore/disinfectant suspension:

Time	1:1x10^1	1:1x10^2	1:1x10^3	1:1x10^4	1:1x10^5
10 min	-	-	TNC	TNC	230
	-	-	TNC	TNC	287
30 min	-	-	TNC	TNC	254
	-	-	TNC	TNC	260
1 hr	-	-	TNC	TNC	146
	-	-	TNC	TNC	124
2 hr	-	TNC	TNC	64	-
	-	TNC	TNC	71	-
4 hr	TNC	72	5		
	TNC	77	6		
6 hr	0	0	0		
	2	0	0		
8 hr	0	0	0		
	0	0	0		

Neutralization Control.

Undiluted	1:1x10^1
TNC	200
TNC	184

IV. DISCUSSION.

Results of the titer showed a viable *B. subtilis* spore concentration of 2.59 x 10^8 spores per ml in the original suspension. Inoculation of 9.9 ml of disinfectant with 100 µl of this suspension produced an initial concentration of 2.59 x 10^5 spores per ml in the assay tube.

Results from these procedures allowed log reductions (LR) and Percent Kill (PK) values to be calculated. They are listed in the following table. Values were computed using the formulas: LR = -Log(S/So) and PK = (1- (S/So)) × 100; where S = concentration of organisms at a specific time; and So = the initial concentration of organisms at time zero. Since there was no significant kill within 30 min, the 10 min data was used for the So values. The 6 hr and 8 hr exposure times did not produce counts high enough to be reliable. Therefore, these data were not used in the linear regressions. Linear regressions

were performed on the log reduction values using the 'fitted line plots' command in the Minitab statistical software package. These graphs are included at the end of this report. The regression equations produced, and the times required to effect a six-log reduction are shown along with the log reduction and percent kill values in the following table.

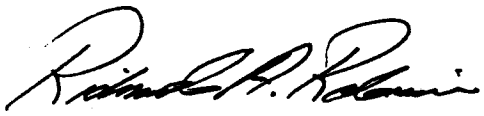
	14 PPM SILVER/1.5% H_2O_2		10 PPM SILVER/1.0% H_2O_2	
Time	LOG REDUCTION	PERCENT KILL	LOG REDUCTION	PERCENT KILL
30 min	-0.03	-7.9	0.003	0.6
1 hr	0.66	78.0	0.28	47.8
2 hr	2.05	99.1	1.58	97.4
4 hr	4.63	99.998	3.54	99.97
Regression Equation:	Y = -0.66704 + 1.32936x		Y = -0.59690 + 1.03933x	
Time for a 6-log reduction:	5.02 hrs		6.35 hrs	

Neutralization control data showed that the disinfectant was adequately neutralized. Expected counts corresponded to those of the titer.

The experimental disinfectant solutions tested exhibited significant sporicidal activity against *B. subtilis* spores. The *B. subtilis* strain used in these evaluations is the same one specified in the AOAC sporicidal test. Spores from this organism represent a significant challenge for most disinfectants The times required to effect a six log reduction are in line with the sporicidal label claims of many cold sterilants.

Test Dates: February 7-8, 2003

By:



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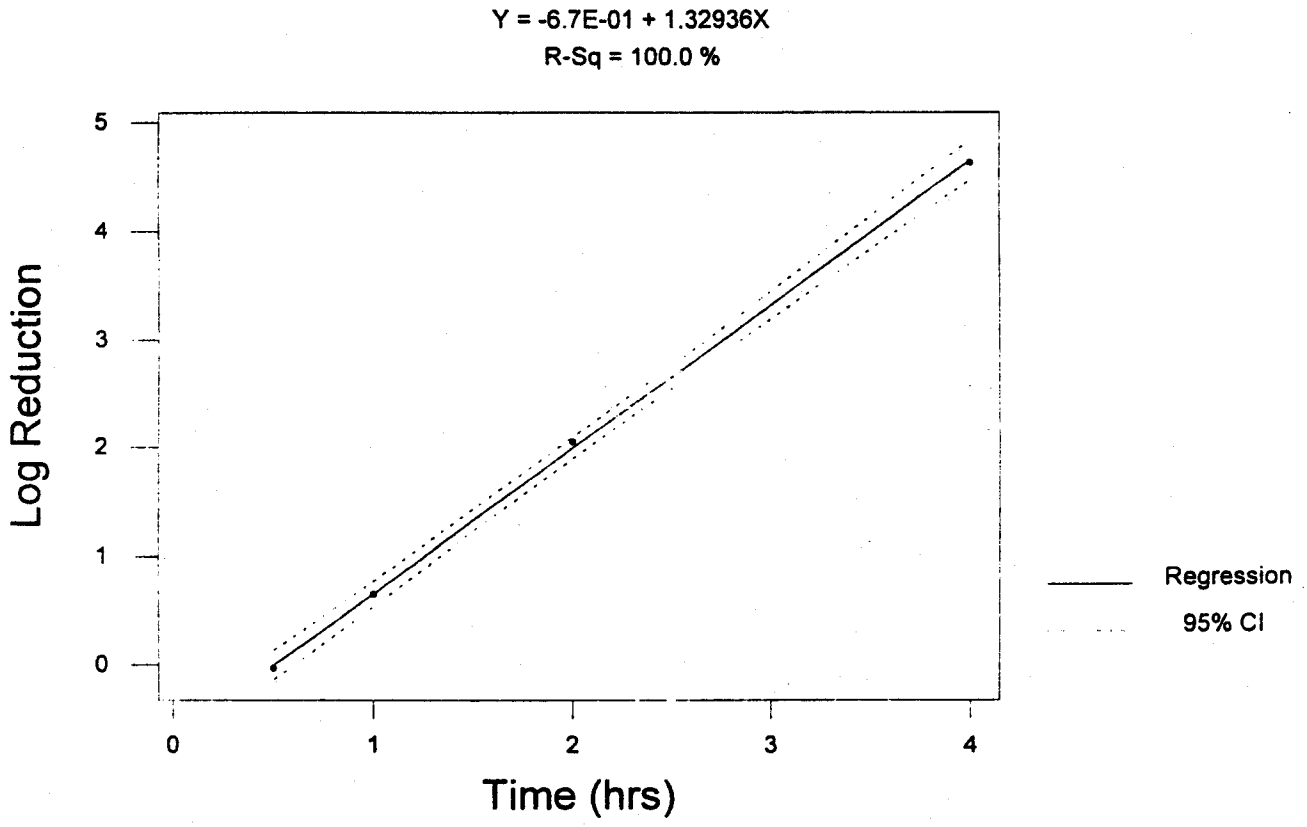
Kill Kinetics of 14 PPM Silver/1.5% H2O2 on B. subtilis Spores



Kill Kinetics of 10 PPM Silver/1.0% H2O2 on B. subtilis Spores