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FINAL STUDY REPORT

INFLUENZA TYPE A VIRUCIDAL PROPERTIES OF A DISINFECTANT PRODUCT MEGA BAC TM PURE OIL USING A SURFACE CARRIER TEST

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SUMMARY

This study was designed to evaluate the virucidal properties of a disinfectant product, Mega Bac TM pure oil, against Human Influenza Virus Type A. The product was tested using 1% with two contact times, ten and sixty minutes and found to cause $\log_{10}$ reductions of 4.4 and 5.1 respectively.

INTRODUCTION

A study was required by NeuMedix Pty Ltd to evaluate a disinfectant product for its virucidal properties against Influenza Type A Virus using ASTM E1053-97. The experimental work was conducted at AMS Laboratories Pty Ltd, 118 Hattersley Street, Rockdale, NSW 2216. AMS Laboratories Pty Ltd are licensed for analysis and testing by the Australian Therapeutic Goods Administration (Licence No. 118118), Australian Pesticides & Veterinary Medicines Authority (Licence No. 6057), and certified by Standards Australia through SAI Global to ISO 9001 – 2000 / ISO 17025 - 1999 (Licence No. QEC 14256).

OBJECTIVE

To determine whether the product, MegaBac, at 1%, exhibits virucidal properties against Influenza Virus Type A in a surface carrier test.

MATERIALS AND METHODS

A. VIRUS STRAIN

The test virus used was Human Influenza Virus Type A, strain FM / 1 / 47. The isolate was obtained from the Institute of Clinical Pathology and Medical Research (ICPMR), Darcy Road, Westmead, New South Wales.

The agent was propagated by inoculation into the allantoic cavity of 9 day embryonated hens eggs. Allantoic fluid was harvested after 72±2 hrs. Only clear allantoic fluid was pooled to form a working seed virus pool. This was checked for sterility, dispensed into approx. 1.8mL aliquots and frozen in liquid nitrogen until use.

B. TEST PRODUCT

One container of product was supplied labelled MEGA BAC TM Pure Oil, Nov 2004 Samples were received on 24/11/2004. The sample was assigned the laboratory reference number 0409829 for initial tests against Influenza A Virus at lower concentrations. For these additional studies at 1%, a new reference number or 0501387 was allocated. The product was prepared in DMSO and placed onto the test carriers for ten and sixty minutes contact times.

C. EXPERIMENTAL DESIGN

The design may be summarized as consisting of application and drying of test virus onto an inanimate surface, followed by standardized application of the test product. Any surviving virus in test or control conditions was assayed using embryonated hens eggs and the production of viral haemagglutinin in the allantoic fluid detected by the use of chicken red blood cells. This design is based upon ASTM E1053 - 97

D. REAGENTS AND SUPPLIERS

1. Phosphate Buffered Saline (PBS) was used for titrating virus suspensions. It was supplied as pre-formulated tablets by Oxoid Australia Pty Ltd and made up as per manufacturers instructions.
2. Medium 199 was supplied by JRH Biosciences Ltd. as single strength, ready to use.
3. Foetal Bovine Serum was supplied by JRH Biosciences Ltd.
4. Chicken Red Blood Cells were supplied by the university of Sydney as aseptically collected chicken blood in Alsever's Solution. They were washed three times in PBS and adjusted to 0.8% v/v before use.
5. DMSO was supplied by MERCK.

E. PREPARATION OF VIRUS HOSTS (EMBRYONATED HENS EGGS)

Nine-day embryonated hens eggs were obtained from Inghams Enterprises Casula Hatchery and transported to AMS Laboratories where they were placed in a 37^{0}C incubator. Eggs were candled to remove any dead or malformed embryos. Suitable eggs had their air-space marked and an inoculation point to avoid the embryo. Marked eggs were placed in egg trays with air-space uppermost and returned to the incubator.

Prior to inoculation, the eggs were swabbed with 70% ethanol and drilled at the inoculation point with an egg drill. The drilled eggs were again swabbed with 70% ethanol and marked ready for use before returning to the incubator.

Eggs were inoculated at 9 days of development.

F. VIRUCIDAL ASSAY

1. 0.2 mL of virus stock was spread over the bottom of sterile glass petri plates covering an area of approximately 28 cm^2. Plates were then air dried in a Class II biohazard cabinet for 20 minutes at ambient room temperature. One plate was left untreated as a toxicity control.
2. Test substance was applied to the plates for the designated exposure times. One plate was left untreated as a dried virus recovery control. Product was allowed to

react with the virus for the designated contact times at ambient room temperature. In this study, contact times of 10 minutes and 1 hour were used.
3. At the designated contact time the reaction was stopped by addition of Medium 199 plus 10% v/v Foetal Bovine Serum and immediately further diluted 10-fold in PBS. These were taken as 10^{-2} and 10^{-3} dilutions respectively.
4. Further 10-fold dilutions were then made by transferring 0.2 mL of each dilution to 1.8 mL of PBS.

G. VIRUS ASSAY

1. Using a sterile 1.0 mL syringe with 21G needle, hosts were each inoculated with 0.2 mL of respective dilutions into the allantoic cavity of 5 embryos per dilution. Five eggs were left uninoculated as viability controls for the population. Five eggs were also inoculated with diluent as negative controls.
2. The inoculation site was swabbed with 70% ethanol, sealed with nail lacquer, and returned to the incubator.
3. Eggs were incubated for a further 3 days. All embryos were again candled 24 hours post inoculation. Any dead embryos were recorded and deemed to be non-specific deaths resulting from the inoculation procedure and were removed from the study.
4. Before commencing the harvest of allantoic fluid the eggs were chilled in a fridge to kill the embryos and contract the blood vessels to enable collection of blood free allantoic fluid.
5. After chilling, the allantoic fluid was collected by insertion of a 21G needle through the inoculation point into the allantoic cavity and withdrawing approximately 0.4 mL allantoic fluid. 0.1 mL was transferred to a designated round-bottomed microtitre well and 0.3 was transferred to a second well (in a separate plate) as a back-up sample in case of further testing requirement.
6. To examine for haemagglutinin activity, serial two-fold dilutions were made to 1:64 and a further 0.1 mL of 0.8% washed chicken red blood cells were added to each well. The plates were gently agitated to mix the red blood cells and left to stand at ambient room temperature for 45 minutes.
7. Each well was then scored for absence of haemagglutination, by observation of a "button" of red blood cells on the bottom of the well (0) or presence, by observation of a uniformly distributed layer of red cells over the bottom of the plate (+).
8. Presence of haemagglutination higher than the 1:32 dilution was taken as evidence of virus replication in the host and recorded accordingly.
9. These reactions were used to determine the Embryo Infective $Dose_{50}$ (EID_{50}) for the different treatment groups by the method of Reed and Muench.

H. CONTROLS

1. Virus. The procedure was performed with dried virus left untreated to determine the titre of surviving virus after maximum contact time.

2. Toxicity. Test substance treated plates were "re-suspended" with Medium 199 and diluted in the same manner as virus treated groups to examine for embryotoxicity of the product after maximum contact time.
3. Product neutralization. Neutralized product was inoculated with approximately 100 EID_{50} of virus to demonstrate adequate product neutralization at the conclusion of the contact times.
4. Host. Five fully prepared eggs were left untreated (no inoculation) to assess the general health of the embryos throughout the assay period. Five embryos were inoculated with DMSO alone. Another five embryos were inoculated with M199 maintenance medium alone.
5. Haemagglutination. Control uninoculated eggs were assayed for presence of non-specific haemagglutination and red blood cells were set up with PBS alone to demonstrate proper button formation.

RESULTS

The untreated FM / 1 / 47 virus control had a $\log_{10}$ titre of 7.5 (Table 1).

The virus remaining after 10 and 60 minutes contact times was $\log_{10}$ 3.1 and 2.4 respectively (Tables 2 and 3). The limit of sensitivity of the assay was a $\log_{10}$ titre of 1.5 (Table 5). Therefore the $\log_{10}$ reductions in the virus titre treated with 1% of the product at 10 and 60 minutes contact times were greater than 4.4 and 5.1 (Table 4).

All embryos inoculated with low titres of virus (10^{-6} dilution) after product neutralization showed haemagglutination activity (Table 6), demonstrating virus infection and thus adequate product neutralization.

The five uninoculated host embryos remained viable and healthy during the course of the experiment (Table 7).

The washed 0.8% chicken red blood cells settled and formed normal "buttons" in the absence of virus.

TABLE 1 INFLUENZA A DRIED VIRUS CONTROL RESULTS.

Virus Dilution	Number Inoculated	Individual Responses (see Note 1)				
10^{-4}	5	+	+	+	+	+
10^{-5}	5	+	+	+	+	+
10^{-6}	5	+	+	+	+	+
10^{-7}	5	+	+	+	+	0
10^{-8}	5	+	0	0	0	0
Total hosts:	25					

Note 1: + represents infected hosts demonstrated by haemagglutination.
0 represents uninfected hosts showing no haemagglutination.
Calculated virus titre = $10^{7.5}$

TABLE 2 RESULTS FOR INFLUENZA A VIRUS TREATED WITH 1% PRODUCT FOR 10 MINUTES

Virus Dilution	Contact time 10 MINUTES										
	No. Inoc.	Individual Responses									
10^{-2}	10	+	0	0	+	+	+	+	+	0	0
10^{-3}	10	+	+	+	+	+	0	+	+	0	+
10^{-4}	5	0	0	0	0	0	-	-	-	-	-
Total hosts:	25										

Note 1: + represents infected hosts demonstrated by haemagglutination.
0 represents uninfected hosts showing no haemagglutination.
- not tested
Calculated virus titre = $10^{3.1}$

TABLE 3 RESULTS FOR INFLUENZA A VIRUS TREATED WITH 1% PRODUCT FOR 60 MINUTES

Virus Dilution	Contact time 60 MINUTES										
	No. Inoc.	Individual Responses									
10^{-2}	10	+	+	+	0	+	+	+	+	+	+
10^{-3}	10	0	0	0	0	0	0	0	0	0	0
10^{-4}	5	0	0	0	0	0	-	-	-	-	-
Total hosts:	25										

Note 1: + represents infected hosts demonstrated by haemagglutination.
0 represents uninfected hosts showing no haemagglutination.
- not tested
Calculated virus titre = $10^{2.4}$

TABLE 4 LOG_{10} REDUCTIONS OF VIRUS AFTER TREATMENT WITH PRODUCT

Treatment	Titre (log_{10})	Reduction (log_{10})
Virus control	7.5	-
1% product (10 min)	3.1	4.4
1% product (60 min)	2.4	5.1

TABLE 5 EMBRYO-TOXICITY STUDY RESULTS

Dilution	No. Inoculated	Individual Responses				
Mega Bac 1% (10^{-2})	5	0	0	0	0	0
M199 mm alone	5	0	0	0	0	0
DMSO alone	5	0	0	0	0	0

0 represents host remained alive and well throughout study.
mm represents maintenance medium

TABLE 6 PRODUCT NEUTRALIZATION STUDY RESULTS

Product Dilution	No. Inoculated	Individual Responses				
Mega Bac 1% 10^{-2}	5	+	+	+	+	+
DMSO alone	5	+	+	+	+	+

+ represents infected hosts demonstrated by haemagglutination.

TABLE 7 DILUENT – INOCULATED CONTROL RESULTS

	No. Inoculated	Individual Responses				
Diluent alone	5	0	0	0	0	0
Total Hosts	5					

0 represents no haemagglutination.

CONCLUSIONS

The study described herein clearly demonstrates that the test product MegaBac Pure Oil at 1% concentration in DMSO was able to kill Influenza A virus at room temperature with contact times of 10 to 60 minutes in a surface carrier test model. The extent of virus kill ranged between 4.4 and 5.1 logs, with a greater kill at the longer contact time.

Signed.....
Paul Priscott, Ph.D., Study Director