Institut Pasteur de Lille

Service d'Expertises en Hygiène Hospitalière

TEST REPORT

DETERMINATION OF VIRUCIDAL ACTIVITY OF PRODUCT NETBIOKEM DSAM

N° Report IPL : NC/0400607M

According to NF EN 14476 on Influenza virus A H1N1

This report concerns only the product subject to the test

I –Principle of the test

The aim of this test is to clarify the viral effect on Influenza A H1N1 virus of one disinfectant
The product is virucidal when there is a 4 log logarithmic reduction of virus titre.

Remarque :
Influenza A H1N1 and Influenza A H5N1 belong to the same family (orthomyxoviridae) and to the same sort (influenza virus type A) of virus. Their difference is uniquely on the glycoprotein structure of hemagglutinine (H1 or H5) ; the neuramidase is the same : "N1".

The realization of this standard comprises several stages, which are :

- Proportion titration of the virus suspension.
- Preliminary test consist in determining the level of disinfecting cytotoxicity according to the cellular line necessary to the detection of the virus. This stage allows defining the cytotoxic concentration of the product, corresponding to the sensitivity of the cells to the tested virus.
- Virus inactivation test by a formaldehyde solution to 0,7 % (m/v). The stage is a control of the test system by the test method.
- Virucidal testing.
- Realization of the virus control.

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II – Identification of the product

Name of product : NETBIOKEM DSAM

Manufacturer : Produits Sanitaires Aeronefs – 19, rue Georges Bidault – 77183 CROISSY BEAUBOURG

Product aspect : yellow

Storage conditions at the laboratories : at laboratory

Test period : 28/03 to 09/06/2007

III – Material and methods

1 – Material

1 – 1 – Reagents and culture media

a) Product submitted to the test (solutions of test)

The product is prepared in hard water at concentrations of 0,125 % - 0,25 % and 0,5 % (v/v)

The product is tested with concentrations of 0,1 % - 0,2 % and 0,4 %

b) Virus :

Influenza virus A H1N1 ATCC-VR-1469 (human)

Used strain is obtained from the American collection of type culture (ATCC, Promochem)

The strain is kept by freezing at -80°C in single usage doses (viral suspension titrating between 10^6 and 10^8 UI/ml).

Strain	Temperature of incubation	Time before the reading
Influenza virus A H1N1	37°C	5 days

c) Cellular line

"MDCK" cells

d) Reagents and media culture

Hard water :

A solution of $CaCO_3$ is prepared according to the standard in such way that the final hardness water of each test tube is lower than 300 mg/l of $CaCO_3$.

Interfering substances :

Clean conditions : Solution of bovine serum albumin (BSA)
The final concentration of bovine serum albumin in the test is 0,3 g BSA per liter.

Medium used for infection :

EAGLE MEM medium + bovine serum albumin + 1M hepes + trypsine

Medium used for the cell cultures :

EAGLE MEM medium + 2 % foetal calf serum + bovine serum albumin + hepes
8 µg/ml

Various agents :

Ethanol diluted at 50 % in ppi water

2 – Testing method

2–1 – Virus titre

Virus titration on monoplayers of cells on microtiters plates

The suspension viral untreated (control and stock suspension) and treated by the product at the test concentration of 0,1 %, 0,2 % and 0,4 %, are diluted in series of 10^{-2} to 10^{-10} maximum, in MEM + 2% FCS frozen. 0,1 ml of each diluted is transferred in 8 wells of the micro titration plate containing the confluent cells, from starting with the highest dilution. After one hour of incubation at 37°C in presence of CO_2 (5 %), 0,1 ml of culture medium is added in each well. The reading of the ECP is realized under the inverted microscope daily after 2 days and 5 days of incubation. Calculation of infectivity titer is determined by the Spearman-Karber method.

2-2 – Determination of subcytotoxic dilution of the disinfectant

The aim of this test is to determine the concentration of chemical disinfectant inducing no sign of toxicity with respect to cellular line allowing the description of the virus to be tested.

Two techniques are described later, and are selected according to following conditions :

The dilution method is the first one tested. It is validated if the difference between the log $TCID_{50}$ of virus titre of the stock suspension and the level non cytotoxic level's is $\geq$ at 5 log. If it's not the case, the molecular sieving with Sephadex is carried out. The validation of the conditions method is similar at the first method. If the results are not satisfactory some is the technique employed, the virus test on disinfecting is unrealizable.

1- Dilution method :

The test solution of product, added with 1/5 of hard water, is diluted in series of 10 to 10 in MEM 2 % FCS frozen. Then 0,1 ml of each dilution is transferred in 8 wells of micro titration plate, we start with the highest dilution. After one hour of incubation at 37°C in presence of CO_2 (5 %), 0,1 ml of culture medium is added in each well. The subcytotoxic effect is assessed after an incubation not exceeding the longest virus culture period cultivated on the system studied (5 days).

2- Molecular sieving technique, with a molecular sievefilter Microspin™S-400 HR columns :

Test solution of the product added with hard water, is filtered on Microspin™S-400 HR columns. Then, filtrate is diluted in series of 10 to 10 in MEM + 2 % FCS frozen. 0,1 ml of each dilution is transferred in 8 wells of micro titration plate. After one hour of incubation at 37°C, with 5 % of CO_2, 0,1 ml of culture medium is added in each well. The subcytotoxicity effect is assessed after an incubation not exceeding the longest virus culture period cultivated on the system studied (5 days).

2-3 – Cell sensitivity to virus

The aim of this test is to make sure that "MDCK" cells with test solution (at the subcytotoxic concentrations) don't alter behavior of virus with the cells. Sensitivity of cells compared to the virus is appreciated by comparison of the virus titre of the stock virus suspension obtained on a cell monolayer treated with the subcytotoxic dilution of disinfectant, with the cell monolayer untreated.

Treatment of cells (with the subcytotoxic concentration of disinfectant)

0,1 ml of the lowest apparently non cytotoxic dilution of the test solution are distributed on to each 8 wells established cell cultures in microtitre plates. Plates are incubated at 37°C for 1 h with 5 % CO_2. In the same time, the stock virus suspension is diluted to 10^{-2} to 10^{-10}. Then, 0,1 ml of each dilution is added in each wells. Plates are again incubated at 37°C for 1 h with 5 % CO_2. 0,1 ml of cell media is added in each wells. The reading of ECP is realized thanks to calculation of infectivity titer are determined by the Spearman-Karber method.

Cells untreated with the subcytotoxic concentration of disinfectant

It's the same procedure as cells treated. Only difference is that the dilution subcytotoxic is removed by MEM 2 % fœtal calf serum. Only these dilutions of the product can be used for the determination of the residual infectivity which produces a titer reduction of the virus of < 1 log.

2-4 – Control of efficiency for suppression of disinfectant activity

Filtration technique

Just after the preparation of the test mixture, reaction is stopped putting 1 ml on the molecular sieving (Microspin^{TM}S-400 HR columns) (10^{-1}).
After a centrifugation, filtrate is diluted in ice cold MEM + 2 % fœtal calf serum from 10^{-2} to 10^{-8}.
Influenza virus A H1N1 is titrated as described in II-2-1.
This procedure has been realized for the test solution at the concentration of 0,4 %

2-5 – Virucidal testing

Test conditions :

Concentrations of the tested solutions : 0,1 % - 0,2 % - 0,4 %

Time of contact : 60 minutes

Remark : Preparation of the test solutions has been realized with hard water

Principle :

The aim of a virucidy test for a disinfectant is to put in contact the viral suspension with an interfering substance and a test solution.
Reaction is stopped after the time of contact notified :

- By dilution on ice cold medium (0,5 ml in 4,5 ml MEM 2 % BCS)
- By filtration technique (molecular sieving with Microspin^{TM}S-400 HR columns). Filtrate is diluted form 10 to 10 in a ice cold medium. Titer of each test is determined as described in II-2-1.

2-6 – Inactivation test of the virus with ethanol 50 % (v/v)

Virucidy tests are realized on the stock suspension of Influenza virus A H1N1 with ethanol in order to control the behavior of our strain with chemical agents.
This control requires realization of the following procedures :

- Control of the ethanol cytotoxicity opposite our cell line
- Control of efficiency of the stopped activity
- Virucidal testing of ethanol (5, 15, 30 and 60 minutes)

2-7 – Titration of the virus standard

The infectivity of the test virus suspension shall be determined under test conditions at contact times 0 min and 60 min.
The product test solution is substituted by water.
Influenza virus A H1N1 is titred as described in II-2-1.

3 – Results : Testing virus of disinfectant NETBIOKEM DSAM on the Influenza virus A H1N1 in 60 minutes

Date of testing : 28/03/2007

3-1 – Titrate and characteristic of viral suspension N

Characteristic of stock virus suspension	*Influenza virus A H1N1*
Virus titrate : Control suspension (log UI/ml)	7

3-2 – Preliminaries tests

- **Cytotoxic dilution of the product NETBIOKEM DSAM and of the filtrate on Microspin^{TM}S-400 HR columns**

Recall of the cellular line : MDCK cells

	Level of cytotoxicity (log)
Dilution technique : C1	6,5
Filtration on Microspin^{TM}S-400 HR columns : C2	1,5

Technique choice :

The select technique have to satisfy the following conditions : N – C ≥ 5 log

Calculation : Only N – C2 is ≥ at 5 log

So the technique by filtration on Microspin^{TM}S-400 HR columns is accepted for the following tests.

3-3 – Sensibility of cells to NETBIOKEM DSAM

Technique realized : filtration
Nontoxic dilution : 10^{-2}

	Treated cells : A	**Untreated cells : B**
Log $TCID_{50}$	6,625	6,5

Conclusion :

Difference between the log of A and B is lower than 1, consequently, the test is validated.

3-3 – Summary table of virucidals tests of the NETBIOKEM DSAM

This table will gather the results obtained by the following points :

- Control of efficiency for suppression of disinfectant activity
- Virucidal testing
- Inactivation test of the virus with ethanol 50 % (v/v)
- Titration of the virus standard

Table of results of product NETBIOKEM DSAM
on Influenza virus A H1N1 under clean conditions

Temperature of test : 20°C

Product	Concentrations	Interfering substance	Level of cytotoxicity	Log $TCID_{50}$ after ..... min					> 4 log reduction after ..... min
				0	5	15	30	60	
NETBIOKEM DSAM	0,1	0,3 g/l SAB	1,5	6,625	n.t.	n.t.	n.t.	<1,5	< 60
	0,2			6,625	n.t.	n.t.	n.t.	<1,5	< 60
	0,4			6,625	n.t.	n.t.	n.t.	<1,5	< 60
Ethanol	50 % (m/v)	PBS	2,5	6,625	5,625	5,25	4,875	3,875	> 60
Virus control	n.a.	PBS	n.t.	6,25	n.t.	n.t.	n.t.	6,5	n.a.
Virus control	n.a.	0,3 g/l SAB	n.t.	6,25	n.t.	n.t.	n.t.	6,25	n.a.

n.a. : not applicable

n.d. : not done

IV – Conclusion

The product NETBIOKEM DSAM lot 02020700 is active at 0,1 % after a contact time of 60 minutes in accordance with NF EN 14476 under clean conditions on Influenza virus A H1N1.

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