UNIVERSITA' DI PISA

Dipartimento di Ricerca Traslazionale
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Pisa, July 24, 2020

To:

KME Italy
Via Morimondo 26
20143 Milano (MI)

Subject: **Declaration of anti-SARS-CoV-2 virucidal activity of Copper and Copper Alloys**

To whom it may concern,

I certify that the Copper and Copper Alloy provided by KME, abates SARS-CoV-2 infectivity of 99.0% when left in contact for one hour at room temperature.

The anti-SARS-CoV-2 activity was determined with three independent experiments, using two sets of material provided at two different times. As detailed in the attached documents, the experiments were performed using a clinical isolate of SARS-CoV-2 that was placed in direct contact with the Copper and Copper Alloy for up to one hour at room temperature. At the end of each period, the virus was collected, diluted 1:10 up to 1000-fold and tested for its capacity to infect and kill the Vero E6 cell line. As shown in the following pages, the test was performed with the limit dilution method, in triplicate, and in compliance with the ISO conditions identifying the proper methods for measuring antiviral activity on various surfaces. Due to the individual sensitivities, the results of SARS-CoV-2 virus might not be applicable for other viruses.

Yours faithfully,

Mauro Pistello

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Methodology

Preparation of the viral stock

A clinical strain of SARS-CoV-2 was isolated from an Italian patient with severe COVID-19 and adapted to grow on Vero E6 cells, a highly sensitive cell line capable of producing a high concentration of viral particles. Propagation was performed up to ten passages and then the propagation was started over with a frozen aliquot. The cytopathic effect was clearly evident after three-four days after seeding and depending on viral input dose. After harvesting, the viral supernatant was centrifuged (400g x 15 min) to remove the cell debris, aliquoted, and stored at -80ºC until use.

For this experiment, three batches of the same clinical isolate were prepared at different times, titrated and diluted to reach 150 tissue culture infectious dose 50% ($TCID_{50}$). Each stock was multiplied on a large scale to obtain a viral suspension having the same characteristics as the standard viral suspension. To minimize risk of possible changes in culture, only 10 passages from the original virus were performed.

Test for cell cytotoxicity of KME materials

Cupper and Cupper Alloys, pre-cut discs of 14 mm diameter, were soaked in 200 μl D-MEM with no serum for one hour at room temperature. Two discs per material were examined. Supernatants were collected from each sample, pooled and added undiluted or 10-fold diluted to a 96-well plate seeded with Vero E6 the day before and cultured in D-MEM + 12.5% serum (serum concentration became 10% after addition of 50 μl medium in contact with the material). Tests were performed in triplicate for each material. Cell viability was assessed by visual inspection with WST assay after four days.

To avoid contamination by bacteria, mold or other microbes possibly present on metal surfaces, disks were soaked in 70% Ethanol at room temperature for 30 min and dried by air flow under a BSL2 cabinet hood.

Test for antiviral activity

Titered viral supernant was diluted to reach the appropriate concentration in D-MEM with no serum and then added to two tissue fragments/each material, 200 μl fragment. Similar procedure was followed with D-MEM. After incubation at room temperature for 5, 10, 30 and 60 minutes, viral supernatants and medium were collected, pooled, and used as such or diluted 1:10 up to 1:1000 (dilutions 50 μl to 500 μl in D-MEM with no serum). Fifty μl each sample was added in triplicate to a 96 well plate seeded with 100,000 Vero E6 cells/well. Plates were prepared the day before and cells were cultivated 150 μl D-MEM 12.5% serum/well. Positive (viral supernatant) was similarly diluted and added in triplicate as such or diluted to the plate. Six wells were supplemented with D-MEM alone, left untreated, and served as negative control. Plates were incubated at 37ºC in 5% humid atmosphere for 4 hours and then the medium was collected from all wells without touching the cells and replaced with fresh D-MEM 10% serum. Plates were incubated for additional three-four days until the cytopathic effect (CPE) was clearly visible.

Presence and extent of CPE were determined by examining the cells with an inverted microscope and the well was scored as virus positive when 75% cells were lysed. To calculate the reduction of infectious viral load, we calculated the Tissue Infectious Dose 50% ($TCID_{50}$) using the Reed and Muench formula.

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Results

Analysis of cell toxicity

Analysis of antiviral activity was preceded by numerous experiments aimed to determine the cytotoxicity of Copper and Copper Alloys. Since the very first experiments it became clear that metals have no detrimental effects to the cells either using the plaque or the viral dilution methods.
We opted to carry out all test with the viral dilution method, largely used for determining the virucidal activity, compliant with ISO legislation, and routinely used in our laboratory to titrate serum neutralizing activity against various viruses including SARS-CoV-2.

Set up of the limiting dilution method to evaluate the antiviral activity

At a difference of the plaque assay, in which the cells are cultivated in semisolid agar and is not possible to change medium, the cells in the limiting dilution method are cultivated in liquid phase thus permitting removal of the medium and interfering agents, if present. To obviate possible detrimental effects caused by harmful substances released by the KME products, we decided to changed the medium four hours after seeding the cell culture with the viral supernatant.

Finding the optimal virus titer for the limiting dilution method

Four hours incubation is a time sufficient for most viruses to attach and enter the cells. We therefore opted for this proceeding but performed additional experiments to check whether replacement of the medium at four hours influences SARS-CoV-2 titer and replication.
Experiments were set in such a way to test in parallel the same viral preparation left until the end of the experiment, i.e. microscope reading, or replaced after four hours with fresh medium. We observed that replacement of the medium did not alter significantly viral infectivity and decided to use 100-150 $TCID_{50}$ as input dose, a manageable viral amount that permitted precise evaluation of antiviral activity of testing material.

Analysis of antiviral activity of KME products

We used a limiting dilution method carried out with Vero E6, an input SARS-CoV-2 dose of 150 $TCID_{50}$ and contact time with Copper and Copper Alloy of 0, 5, 10, 30, and 60 minutes. The medium in contact with materials was replaced four hours after with fresh medium and cells further incubated until visual inspection (four days after seeding).
Discs of 14 mm diameter of KME products were placed in a 24 well plate. For each experiment we used four discs, two that were soaked with the titered virus supernatant, and two with fresh medium. Each disc was submerged in 200 μl, a volume sufficient to submerge and get in contact with the disc. At 0, 5, 10, 30 and 60 minutes the medium was collected, pooled and used as such or 10-fold diluted until 1:1000. As mentioned, the medium was replaced with fresh medium four hours later.
The antiviral activity was assayed in three independent experiments using two batches of Copper and Copper Alloys provided by KME. In the three experiments performed, we obtained very similar results that are shown cumulatively in the enclosed document and expressed as reduction of $TCID_{50}$ and log $TCID_{50}$ in the Table below.
Both materials showed a very strong virucidal activity and Copper slightly outperformed Copper Alloys in a nice and consistent time-dependent scale. As shown in the table below, reduction was noticeable since the first time examined, five minutes, increased three times with only five further

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minutes of incubation and eliminated over 95% viral infectivity in 30 minutes. At one-hour contact the Copper Alloy eliminated over 99% virus and Copper totally abated the viral titer, to such an extent that the residual virus was capable to lyse the cells in only 2/18 wells. The same number of infected wells was obtained with the untreated viral stock diluted 1:1000 (see attached datasheet). This result indicated that Cupper abates viral infectivity of about three logs in one hour.

Residual SARS-CoV-2 infectious dose following contact with KME Copper and Copper Alloys

		Contact time *(minutes)*				
		0	5	10	30	60
Copper	**$TCID_{50}$**	154.8817	66.0693	14.4544	3.1623	0.0003
	% reduction $TCID_{50}$[a]		**57.34**	**90.67**	**97.96**	**100.00**
	Log $TCID_{50}$	2.19	1.82	1.16	0.50	-3.50
	% reduction Log $TCID_{50}$[a]		**16.89**	**47.03**	**77.17**	**259.82**
Copper Alloy	**$TCID_{50}$**	154.8817	87.0964	23.9883	6.6069	0.1585
	% reduction $TCID_{50}$[a]		**43.77**	**84.51**	**95.73**	**99.90**
	Log $TCID_{50}$	2.19	1.94	1.38	0.82	-0.80
	% reduction Log $TCID_{50}$[a]		**11.42**	**36.99**	**62.56**	**136.53**

[a] *% reduction compared to viral stock*

Conclusions

KME Copper and Copper Alloy possess strong antiviral activity against SRAS-CoV-2, COVID-19 etiological agent. To appreciate the virucidal activity in full, testing with higher viral doses is advisable to evaluate whether reduction of infectivity goes beyond the two-three logs found herein.

Reed and Muench Spreadsheet

Stock virale SARS-CoV-2 *(materiale di riferimento)*

TCID50	Single Wells		Cumulative wells		Total	% CPE		
	CPE	No CPE	CPE	No CPE				
100	18	0	48	0	48	100,00	**Diff of logs**	0,19
10	18	0	30	0	30	100,00	**Log_{10} 50%**	**2,19**
1	10	8	12	8	20	60,00	**Titolo**	**154,8817**
0,1	2	16	2	24	26	7,69		

Ottone 5 minuti

TCID50	Single Wells		Cumulative wells		Total	% CPE		
	CPE	No CPE	CPE	No CPE				
100	18	0	46	0	46	100,00	**Diff of logs**	-0,06
10	18	0	28	0	28	100,00	**Log_{10} 50%**	1,94
1	9	9	10	9	19	52,63	**% dim Log***	**11,42**
0,1	1	17	1	26	27	3,70	**Titolo**	**87,0964**
							% dim. Tit.*	**43,77**

Ottone 30 minuti

TCID50	Single Wells		Cumulative wells		Total	% CPE		
	CPE	No CPE	CPE	No CPE				
100	18	0	25	0	25	100,00	**Diff of logs**	0,82
10	7	11	7	11	18	38,89	**Log_{10} 50%**	0,82
1	0	18	0	29	29	0,00	**% dim Log***	**62,56**
0,1	0	18	0	47	47	0,00	**Titolo**	**6,6069**
							% dim. Tit.*	**95,73**

Rame 5 minuti

TCID50	Single Wells		Cumulative wells		Total	% CPE		
	CPE	No CPE	CPE	No CPE				
100	18	0	43	0	43	100,00	**Diff of logs**	0,82
10	18	0	25	0	25	100,00	**Log_{10} 50%**	1,82
1	7	11	7	11	18	38,89	**% dim Log***	**16,89**
0,1	0	18	0	29	29	0,00	**Titolo**	**66,0693**
							% dim. Tit.*	**57,34**

Rame 30 minuti

TCID50	Single Wells		Cumulative wells		Total	% CPE		
	CPE	No CPE	CPE	No CPE				
100	16	2	18	2	20	90,00	**Diff of logs**	0,50
10	2	16	2	18	20	10,00	**Log_{10} 50%**	0,5
1	0	18	0	36	36	0,00	**% dim Log***	**77,17**
0,1	0	18	0	54	54	0,00	**Titolo**	**3,1623**
							% dim. Tit.*	**97,96**

**** Diminuzione rispetto allo stock virale***

Ottone 10 minuti

TCID50	Single Wells		Cumulative wells		Total	% CPE		
	CPE	No CPE	CPE	No CPE				
100	18	0	33	0	33	100,00	**Diff of logs**	0,38
10	13	5	15	5	20	75,00	**Log_{10} 50%**	1,38
1	2	16	2	21	23	8,70	**% dim Log***	**36,99**
0,1	0	18	0	39	39	0,00	**Titolo**	**23,9883**
							% dim. Tit.*	**84,51**

Ottone 60 minuti

TCID50	Single Wells		Cumulative wells		Total	% CPE		
	CPE	No CPE	CPE	No CPE				
100	5	13	5	13	18	27,78	**Diff of logs**	-0,80
10	0	18	0	31	31	0,00	**Log_{10} 50%**	-0,8
1	0	18	0	49	49	0,00	**% dim Log***	**136,53**
0,1	0	18	0	67	67	0,00	**Titolo**	**0,1585**
							% dim. Tit.*	**99,90**

Rame 10 minuti

TCID50	Single Wells		Cumulative wells		Total	% CPE		
	CPE	No CPE	CPE	No CPE				
100	18	0	30	0	30	100,00	**Diff of logs**	0,16
10	9	9	12	9	21	57,14	**Log_{10} 50%**	1,16
1	3	15	3	24	27	11,11	**% dim Log***	**47,03**
0,1	0	18	0	42	42	0,00	**Titolo**	**14,4544**
							% dim. Tit.*	**90,67**

Rame 60 minuti

TCID50	Single Wells		Cumulative wells		Total	% CPE		
	CPE	No CPE	CPE	No CPE				
100	2	16	2	16	18	11,11	**Diff of logs**	-3,50
10	0	0	0	16	16	0,00	**Log_{10} 50%**	-3,5
1	0	18	0	34	34	0,00	**% dim Log***	**259,82**
0,1	0	18	0	52	52	0,00	**Titolo**	**0,0003**
							% dim. Tit.*	**100,00**