Test report C14ML1760BC

Evaluation of the effectiveness of

novel green/white nylon copper infused fabric

Test virus: bovine coronavirus (BCV) (surrogate of human coronavirus)

Method: In-House method following JIS Z 2801 and ASTM E 2180 tests for the evaluation of virucidal activity of treated materials

Sponsor:
Copper Clothing Limited
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1. Identification of test laboratory

Dr. Brill + Partner GmbH Institute for Hygiene and Microbiology, Norderoog 2, DE-28259 Bremen

2. Identification of sample

novel green/white nylon copper infused fabric and Tork Premium Spezial Tücher, article no. 90491, SCA Tork (reference)

3. Materials

3.1 Culture medium and reagents

- Eagle's Minimum Essential Medium with Earle's BSS (EMEM, Biozym Scientific GmbH, catalogue no. 880121)
- Earle's balanced salt solution (EBSS, Biochrom AG, article no. L1915)
- Fetal calf serum (Thermo Fisher, article no. CH30160.02)
- Aqua bidest. (Fresenius Kabi Deutschland, article no. P2N 1636071)
- PBS (Invitrogen, article no. 18912-014)
- BSA (Sigma-Aldrich-Chemie GmbH, article no. CA-2153)

3.2 Virus and cells

The human BCV strain L9 was obtained by Dr. G. Zimmer, Institute of Virology at the School of Veterinary Medicine Hannover (Tierärztliche Hochschule, D-30559 Hannover).

The U373 cells (passage 12) were as well obtained by Dr. G. Zimmer, Institute of Virology at the School of Veterinary Medicine Hannover (Tierärztliche Hochschule, D-30559 Hannover).

The cells were inspected regularly for morphological alterations and for contamination by mycoplasmas. No morphological alterations of cells and no contamination by mycoplasmas could be detected.
3.3 **Apparatus, glassware and small items of equipment**

- CO₂ incubator, Nunc GmbH & Co. KG, model QWI 350
- Agitator (Vortex Genie Mixer, type G 560E)
- Centrifuge (Sigma-Aldrich-Chemie GmbH, type 113)
- Microscope (Olympus, type CK 30)
- Centrifuge 5804 R (Eppendorf AG)
- Adjustable and fixed-volume pipettes (Eppendorf AG)
- Polystyrol 96-well microtitre plate (Nunc GmbH & Co. KG, Wiesbaden, Germany)
- Cell culture flask (Nunc GmbH & Co. KG, Wiesbaden, Germany)
- Sealed test tubes (Sarstedt AG & Co., Nümbrecht, Germany).

4. **Experimental conditions**

Sponsor-provided antimicrobial material (novel green/white nylon copper infused fabric) and non-antimicrobial towel (Tork Premium Spezial Tücher, Art. No. 90491, SCA Tork, SCA Hygiene Products AFH Sales GmbH) as control (reference) were inoculated with a virus inoculum (test virus suspension + interfering substance) and later assayed for evaluation of effectiveness in a recovery process for virus infectivity by cell culture.

Here, the antimicrobial properties of the material supplied by Copper Clothing Limited were tested against bovine corona virus (BCV) with an In-House method of Dr. Brill + Partner GmbH being a combination of elements of the Industrial Japanese Standard JIS Z 2801 and the ASTM E 2180-07 due to the fact there are no test methods for viruses.

5. **Methods**

5.1 **Preparation of test material**

For the preparation of the material pieces of 1 x 1 cm (both materials) were cut in sterile conditions and after a folding step transferred in an Eppendorf cup. By this procedure it was ensured that the whole virus inoculum came into contact with antimicrobial material and the reference.

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5.2 Preparation of test virus suspension

For preparation of test virus solution, U373 cells were cultivated in a 75 cm² flask with in EMEM supplemented with L-glutamine, non-essential amino acids and sodium pyruvate and 10 % fetal calf serum. Before virus infection, cells were washed three times with phosphate buffered saline (PBS), incubated for 3 h with EMEM without FCS and were washed once with EMEM supplemented with trypsin. For virus production, BCV strain L9 was added to the prepared monolayer. After an incubation period of 24 to 48 hours cells were lysed by a rapid freeze/thaw cycle. Cellular debris was removed by low speed centrifugation and the supernatant was directly used as the test virus suspension.

5.3 Infectivity assay

Infectivity was determined by means of end point dilution titration using the microtitre process. For this, samples were immediately diluted at the end of the exposure time with ice-cold EMEM with trypsin and 100 µl of each dilution were placed in eight wells of a sterile polystyrene flat bottomed plate with a preformed U373 monolayer. Before addition of virus, cells were washed once with EMEM and incubated for 3 h with 100 µl EMEM with trypsin. Incubation was at 37 °C in a CO₂-atmosphere (5.0 % CO₂ - content). Finally, cultures were observed for cytopathic effects for six days of inoculation. The infectious dose (TCID₅₀) was calculated according to the method of Spearman (2) and Kärber (3) with the following formula:

\[
\log_{10}TCID_{50} = X_0 - 0.5 + \sum \frac{r}{n}
\]

meaning
\[X_0 = \log_{10} \text{of the lowest dilution with 100 % positive reaction}
\]
\[r = \text{number of pos. determinations of lowest dilution step with 100 % positive and all higher positive dilution steps}
\]
\[n = \text{number of determinations for each dilution step.}
\]

5.4 Calculation and verification of virucidal activity

The virucidal activity of the treated material was evaluated by calculating the decrease in titre in comparison with the virucidal activity of the non-treated material.

5.5 Inactivation assay

In the beginning titres of the virus inoculum were determined (in duplicate). These values were needed as base line.
This was followed by the contamination assay: 10 µl of the test virus suspension (mixed with soil load 1:1, clean conditions = virus inoculum) are deposited on the treated and non-treated materials followed by an immediate recovery after deposition. For this, carriers were deposited in 990 µl EBSS and tested for infectivity. This assay controls whether a recovery is possible after a short contact with treated and non-treated materials without reduction of virus titre.

Furthermore, for controls of cytotoxicity materials were contaminated with 10 µl Earle’s Balanced salt solution instead of test virus suspension and recovered in 990 µl eluent (EBSS) (no virus, effect of the treated and non-treated materials on the permissive cells for detection of virus needed). This control shows the cytotoxicity of both materials used.

Another control was done for the evaluation of the drying process. Here the contamination of the treated and non-treated materials after a drying process of the virus inoculum is controlled (drying process 10 minutes). These data are needed as baseline (determination of loss of virus inoculum during the drying process).

10 determinations in parallel for each material (challenge assay) in order to see any difference between non-treated and treated materials (fixed time provided by the sponsor of the study: 60 minutes). Elution was immediately done with EBSS.

Determination of virus titres by end point dilution method (see 5.2)

6. Results

Results of examination are shown in tables 1 to 2. Table 1 shows the controls whereas table 2 demonstrates the comparative titration of the eluant after the recovery process.

The titre of the virus inoculum was 6.00 ± 0.38 and 5.63 ± 0.25 (mean ± 0.23). The titres of the virus inoculum after contact with the antimicrobial material and the reference were 5.88 ± 0.37 and 5.38 ± 0.49 (novel green/white nylon copper infused fabric) and 5.63 ± 0.45 and 6.00 ± 0.55 (reference), respectively showing no loss of virus titre in both cases.

After the drying process (10 min) the following titres were observed: ≤ 1.88 ± 0.37 and ≤ 1.50 ± 0.00 (novel green/white nylon copper infused fabric, mean: ≤ 1.69 ± 0.19) and 4.63 ± 0.25 and 4.75 ± 0.33 (reference, mean: 4.69 ± 0.21), respectively.
After a contact time of 60 minutes only on one material residual virus could be measured with the novel green/white nylon copper infused fabric (Table 2). In contrast, examining the non-treated materials residual virus could be detected in all cases. The following mean values resulted: ≤1.55 ± 0.04 (novel green/white nylon copper infused fabric) and 2.98 ± 0.12 (reference). A difference of 1.43 log₁₀ steps between both materials was visible based on the 10 fold determinations after 60 minutes exposure time.

Due to cytotoxicity the lower detection limit was ≤ 1.50 log₁₀CD₅₀/ml with the novel green/white nylon copper infused fabric (cytotoxic in the undiluted eluat) and 0.50 log₁₀CD₅₀/ml with the reference (non-cytotoxic in the undiluted eluat).

In summary, the difference observed between the materials tested is based on the inactivation process during the 10 minutes drying process (reduction factor of 3.94 log₁₀ of the treated material and 1.13 log₁₀ of the reference) and on the behaviour of the materials during the challenge time of 60 minutes (mean virus titre ≤ 1.55 ± 0.04 versus 2.98 ± 0.12)

Bremen, 02.10.2014

- Dr. Jochen Steinmann -
Scientific Director

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7. Quality control

The Quality Assurance of the results was maintained by performing the determination of the virus-inactivating properties of the disinfectant in accordance with Good Laboratory Practice regulations:


8. Records to be maintained

All testing data, protocol, protocol modifications, the final report, and correspondence between Dr. Brill + Partner GmbH and the sponsor will be stored in the archives at Dr. Brill + Partner GmbH.

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The test results in this test report relate only to the items examined.
9. Literature

1. ASTM E 2180 -01: Standard Test Method for Determining the Activity of Incorporated Antimicrobial Agent(s) In Polymeric or Hydrophobic Materials

2. JIS Z 2801:2000: Antimicrobial products — test for antimicrobial activity and efficacy


Appendix:

Legend to the Tables

Table 1: Virus titres of the controls and cytotoxicity of both products

Table 2: Virus titres (bovine coronavirus) in the 10fold assay with treated (novel green/white nylon copper infused fabric) and non-treated material (Tork Premium Spezial Tücher) after 60 minutes exposure time
Table 1: Virus titres of the controls and cytotoxicity (3667)

<table>
<thead>
<tr>
<th>Control</th>
<th>Interfering substance</th>
<th>log\textsubscript{10} TCID\textsubscript{50}/ml</th>
<th>log\textsubscript{10} CD\textsubscript{50}/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>test virus inoculum</td>
<td>-</td>
<td>6.00±0.38</td>
<td>5.63±0.25</td>
</tr>
<tr>
<td>virus titre after contact with the copper material (t_0)</td>
<td>clean conditions</td>
<td>5.88±0.37</td>
<td>5.38±0.49</td>
</tr>
<tr>
<td>virus titre after contact with the reference (t_0)</td>
<td>clean conditions</td>
<td>5.63±0.45</td>
<td>6.00±0.55</td>
</tr>
<tr>
<td>virus titre after drying on copper material (t_{10})</td>
<td>clean conditions</td>
<td>≤1.88±0.37</td>
<td>≤1.50±0.00</td>
</tr>
<tr>
<td>virus titre after drying on the reference (t_{10})</td>
<td>clean conditions</td>
<td>4.63±0.25</td>
<td>4.75±0.33</td>
</tr>
<tr>
<td>cytotoxicity copper material of the eluat</td>
<td>clean conditions</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>cytotoxicity reference of the eluat</td>
<td>clean conditions</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

n.d. = not done

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Table 2: Virus titres (bovine coronavirus) in the 10fold assay with treated (novel green/white nylon copper infused fabric) and non-treated material (reference: Tork Premium Spezial Tücher) after 60 minutes exposure time (3667)

<table>
<thead>
<tr>
<th>Product</th>
<th>carriers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>copper material</td>
<td>≤1.50 ± 0.00</td>
</tr>
<tr>
<td>reference</td>
<td>2.83 ± 0.37</td>
</tr>
</tbody>
</table>

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