ANTIVIRAL TEXTILES AND ANTIVIRAL ACTIVITY TESTING - THE USE OF BACTERIOPHAGE SURROGATE FOR ANTIVIRAL ACTIVITY TESTING

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ABSTRACT

The risk of dissemination of highly contagious viral diseases (as COVID-19, Ebola) led in the increasing need to develop functional textiles and surfaces with antiviral effect. Antiviral textiles are designed to reduce the viability and infectivity of viruses on their surfaces and by this way to reduce the cases of infection (including re-infection or cross-infection with contaminated textiles). Different antiviral agents and diverse techniques of their application are used for functionalized textiles manufacturing. The most often used antivirals are metallic and ionic silver and copper, iron oxide, quaternary ammonium salts. The aim of the process is to prepare textiles with long-term durable finishing effective in viral activity inhibition. The basic step of functionalized antiviral textiles development is antiviral effectivity testing. The safe method of testing with the use of Phi6 bacteriophage, SARS-CoV-2 and Ebola virus surrogate, was modified for antiviral textiles testing. The samples of textiles with antiviral finishing were tested by the bacteriophage-based method and excellent antiviral activity was detected for all tested materials. The woven cotton was used as reference untreated material, the different textile cotton structures with similar square weight were compared and no statistically significant difference was found between the resulting antiviral efficacy values. A simple and quickly feasible screening method for determining the antiviral properties of textiles, especially with leaching-type of treatment, was also designed and tested.

KEYWORDS

Antiviral; Bacteriophage; Finishing; Textiles; Virus.

INTRODUCTION

The fast spread of the COVID-19 virus in the last years together with continuous emergence of other infectious diseases (SARS, H1N1 influenza, Ebola haemorrhagic fewer), bring the highest need for development and testing of antiviral textiles [1] [2]. Textiles help to protect human from un-favorable conditions of environment, but for protection from microorganisms and viruses they need to be treated by antimicrobial finishings. Antiviral treatment of the personal protection equipment and the textiles used in public space can reduce the risk of viral infection transmission due to the contact with contaminated textiles and objects [3-5]. For this reason, there is a growing need for the development of efficient and accessible methods of antiviral activity testing, and hand in hand with them to optimize the testing methods as well.

The mainly used strategy for producing antiviral and antipathogenic textiles, in general, is physically load additives on the surface of the fabric and/or microfibers, although new fabrication methods are available too (copper ion-textile based on fundamentally different principle of incorporating copper ions into the cotton structure at the molecular level described by Quian et al. [6]). Antiviral chemicals (inorganic metals and metal nanoparticles, such as silver, zinc or copper, quaternary ammonium compounds or organic compounds with antiviral properties [5] [7] can be applied to textiles through various chemical finishes. Surface modifications (microencapsulation, plasma treatment, electrospinning) can help to improve durability and provide long-lasting effect of antiviral or generally antipathogenic treatment [4] [5] [8]. The selection of a particular method depends on factors such as the desired level of antiviral activity, fabric type, intended use, cost, and regulatory considerations [4].

Independently on the antiviral compound, the method of antiviral textile production and the way of the use, there is a need for the antiviral effectivity testing in the

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course of antiviral fabrics development. Specific viruses are chosen for antiviral effectivity testing, depending on the use of textile product and the availability of the testing method. The international standard ISO 18184 "Textiles - Determination of antiviral activity of textile products." [9] mentions the use of human influenza virus as a representative of enveloped viruses, and feline calicivirus as a representative of non-enveloped viruses. The use of another evaluated virus is possible. The testing virus could be a carefully chosen surrogate virus, used as a substitute for specific human viruses. Surrogate viruses possess similar properties to their human counterparts, allowing researchers to study antiviral activity in a controlled laboratory setting [10]. Calicivirus or Vaccinia virus are commonly used as a surrogate or model viruses in antiviral testing. Bacteriophages are the promising biosafety level 1 (BSL-1) substitutes of human viruses, due to their resistance towards antimicrobials, environmental conditions and antiviral compounds [11-13]. The Phi6 bacteriophage (double-stranded RNA enveloped virus) has been used in a wide range of testing applications as a valuable surrogate of human enveloped viruses, such as SARS-CoV-2 virus, influenza virus and Ebola virus, due to their lipidic surface structures [2] [3] [12] [14]. As safe infectious testing particle enables testing in conditions where the use of an animal virus would not be safe [1] [2]. The example of the use are materials for face masks and the testing of antiviral effectivity and viral filtration efficiency with the same virus [13].

Due to the limited availability of antiviral activity testing, our work was focused on the development and application of an effective and affordable method for testing the antiviral activity of textile materials. Based on the previously published data and the comparison of structural properties of viruses, the bacteriophage phi6 was chosen as a suitable enveloped surrogate virus for the determination of antiviral activity of functionalized textiles. The aim of this work was to optimize the bacteriophage-based method for determination of antiviral activity of functionalized textiles and overcome the limitations of testing with selected animal viruses (lower titre of particles in suspension, sensitivity to environmental conditions, costs of the testing). The method is based on the common principle of assessment of antiviral activity of textiles - the viral suspension test. In this test, the textile sample is directly exposed to a suspension of the target virus. After a specified contact time, the virus is recovered from the textile, and its infectivity is assessed. The different testing system allows to extend the method by qualitative preliminary test, which was designed besides the quantitative testing.

The resistance of functional adjustments, including antiviral treatments, during using and maintenance of textiles, is important characteristic of treated products. The leaching of antimicrobial agents is important for durability of the treatment and the kind of antimicrobial effect. A non-leaching type of the functionalized textile is generally preferred for clothing as it may retain its activity for a longer time. The leaching type of the treatment is used for medical textiles and disposable materials, the leaching substances are characterized by stronger, biocidal, antimicrobial activity [4]. The bacteriophage-based method of antiviral activity determination can give preliminary and fast information about leaching of the textile treatment.

MATERIALS AND METHODS

Bacteriophage Phi6 DSM 21518 and the host strain *Pseudomonas* sp. DSM 21482 (testing virus and their host) were purchased from German collection of microorganisms (Deutsche Sammlung fur Mikroorganismen und Zellkulturen, DSMZ) – Leibnitz Institute, Braunschweig, Germany. Tryptic soya broth (TSB) and Tryptic soya agar (TSA) for the host strain cultivation, Soybean casein digest medium with Tween 80 and Lecithin (SCDLP) and Tris-buffer base were purchased from HiMedia.

A Phi6 stock solution of 10^{10} plaque forming units (PFU)/ml of TSB was used for the virus propagation and for testing procedure. Liquid lysis method described by DSMZ [15] was used for bacteriophage propagation. Double-layer plaque assay for the infected cells number determination was provided according to Plohl [3] with minor modifications. There were added 3 ml of top agar on the standard 90 mm Petri plate with bottom agar (TSA), and 0.1 ml of host bacteria suspension and 0.1 ml of diluted sample of bacteriophage suspension. SM-buffer [15] was used for bacteriophage dilution. The plates with inoculated top-agar were allowed to solidify and then were cultivated at 25°C. The infectivity titre of the virus was determined as a number of plaque-forming units per ml of suspension [16] from the number of plaques (clear zones) on the plates after 24 hours of cultivation.

Four knitted fabrics of different composition (Table 1(a)) with the same antiviral silver-based treatment harmonized with current biocidal regulation and two woven cotton reference materials (Table 1(b)) were used in this study. All tested materials were manufactured by SINTEX. The negative untreated reference samples of unbleached woven cotton were washed in 10 cycles without detergent at 60°C (10 min followed by 2-time rinsing for 5 min) [9].

Determination of antiviral activity

The test specimens with mass of (0.40 ± 0.05) g were prepared in triplicates and cut on the (20 x 20) mm pieces. The vial containers were used for sterilization of samples in the autoclave at 121°C and 103 kPa for 15 min. The triplicates of control untreated specimens were prepared by the same way, as well as specimens for cytotoxicity testing [9].

Table 1a. The antiviral materials used for the bacteriophage-based method optimization. The same antiviral finishing agent (with preliminary validated antiviral effect) was used for all materials.

	Sample 1	**Sample 2**	**Sample 3**	**Sample 4**
Material structure/colour	single jersey white	doubleface blue	elastic single jersey black	single jersey light blue
Material composition	50 % cotton 50 % polyester	51 % polyester 49 % cotton	90 % polyester 10 % Lycra	100 % cotton
Mass per unit area [g/m^2]	158	185	230	131
Width [cm]	176	200	150	163

Table 1b. Reference untreated materials used for the bacteriophage-based method optimization. Woven unbleached cotton was used as reference material for antiviral activity determination.

	736A	737A
Material composition	100 % cotton	100 % cotton
Mass per unit area [g/m^2]	113	103
Warp sett [cm^{-1}]	26	39
Weft sett [cm^{-1}]	23	27

Infectivity titre test was used for antiviral activity of textile samples determination. Antiviral activity of the tested products was evaluated on the base of antiviral efficacy value, *Mv*.

The **antiviral efficacy value** was calculated as follows:

$$Mv = lg\,[Va] - lg\,[Vc], \quad (1)$$

where Mv is the antiviral efficacy value, *lg* [Va] is the common logarithm average of 3 infectivity titre value immediate after inoculation of untreated specimen, *lg* [Vc] is the common logarithm average of 3 infectivity titre value after 2 h contacting with the treated specimen.

The infectivity titre of control specimens (untreated) was determined immediately after inoculation and after contact time (2 h). Logarithm reduction value of infective titre of control specimens was less than 1.0.

At the beginning of every test, 200 μl of the virus suspension (10^7 infection particles per ml, last dilution in sterile distilled water) was applied on the textile test specimen and untreated (reference) sample. After contact time 2 hours (or immediately in the time 0), the virus was recovered from the specimens, 20 ml of sterile washing out solution - SCDLP medium – was added and vials were agitated by Vortex mixer 5 times for 5 s Medium with recovered virus was diluted and appropriate dilutions were inoculated to the top agar, together with the host cells, for infectivity titre determination (Fig. 1).

The double-layer agar plaque assay provides a measure of the viability of viral particles present in a sample, as each plaque corresponds to a single infectious unit. Antiviral efficacy value is calculated as the reduction of viral infectivity by treated textile in comparison with untreated reference sample [9].

Agar diffusion assay

Fabric specimens were cut to squares (2 x 2 cm approx.) and placed on the top TSA agar pre-inoculated by *Pseudomonas* sp. DSM 21482 and bacteriophage Phi6 DSM 21518 (double layer agar in a Petri dish was used). Plates were incubated at 25°C for 24 h. The zones of inhibition were checked visually and microscopically (if no zone was detected). The assay allows to characterize antiviral textile as leaching or un-leaching type. Leaching antiviral agents diffuse to agar surrounding the sample and inhibit the infection process, resulting in inhibition zone appearance.

RESULTS AND DISCUSSION

Four samples of different material composition, with mass per unit area (131 – 230) g/m^2, treated by the same antiviral compound, were tested for antiviral effectivity by bacteriophage-based method of antiviral textile testing. Two untreated control fabrics (100% cotton, unbleached, 10 times washed) of different woven parameters were used for testing and *Mv* calculation (Table 1(a,b)).

Sample 1 was white single jersey polyester/cotton 50/50 %. Sample 2 was blue doubleface 49 % polyester, 51 % cotton, sample 3 was black elastic single jersey 90% polyester, 10 % Lycra, and sample 4 was light blue single jersey, 100 % cotton. All samples were treated by identical antiviral compound of the same concentration. The reference cotton infectivity titre values were used for the antiviral efficacy value determination. No toxic effect of antiviral textile samples toward host cells has been demonstrated by control test. The infectivity titre of viral suspension used for testing was 3 – 5 x 10^7 PFU/ml. The logarithm reduction values of infective titre of control specimens were less than 1, what is the condition of valid test for 2 h contact time concerning ISO 18184 [9].

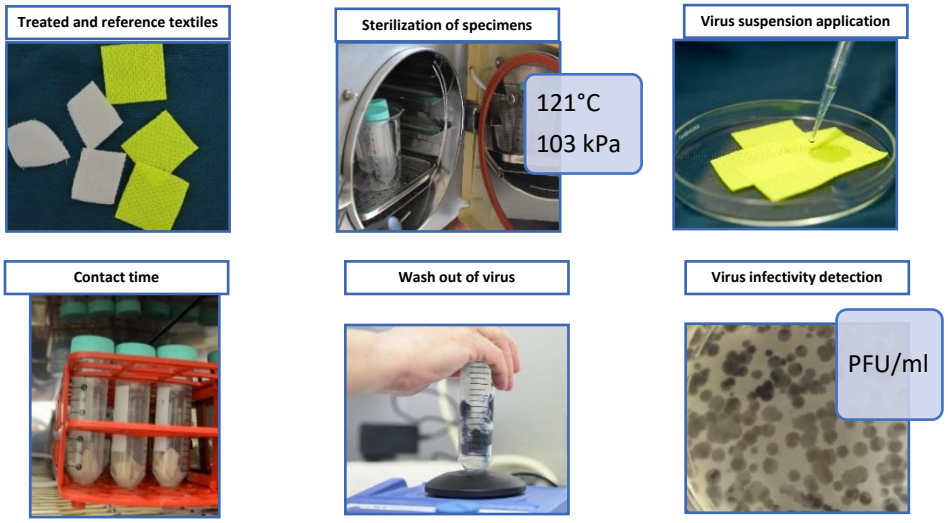

Figure 1. Determination of antiviral efficiency of textile products by the use of bacteriophage-based method. Illustration photos show sample preparation and treatment, virus application and recovery and resulting bacteriophage plaques in top agar.

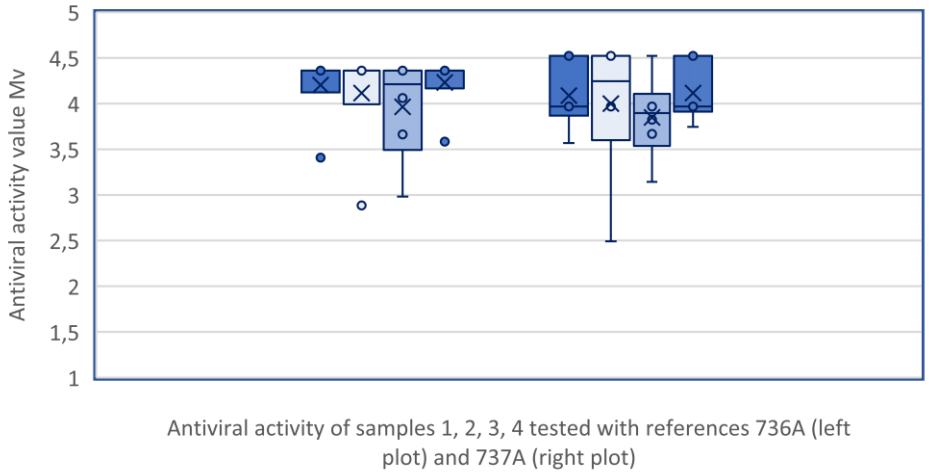

Figure 2. Boxplots of antiviral activity (*Mv*) results distribution. 6 *Mv* values were determined for each sample (1 – 4) with reference 736A and 6 *Mv* values with reference 737A. Average Va value of *lg* (PFU) was taken for Mv calculation.

Table 2a. Antiviral activity values (*Mv*) of treated samples (*lg* reduction Va/Vc, PFU/ml). Outliers were excluded from dataset used for calculation.

	Mv			
	Sample 1	Sample 2	Sample 3	Sample 4
Reference 736A	4.4	4.4	4	4.4
Reference 737A	4	4	3.8	4.1

The samples were compared on the base of *Mv* value, calculated as described in methods part. This value is important for evaluation of effectivity of antiviral treatment (categories according to Annex F of ISO 18184 standard [9]). The two available reference woven cotton materials were used with the aim to confirm or exclude the effect of reference fabric structure on antiviral activity results.

The Shapiro-Wilk test for some of sample results gave p-values by order of magnitude lower than p = 0.05, which means, that null hypothesis of normality was rejected and some of datasets were non normal distributed. That's why we used boxplots for identification of outliers, where the box ranges from the first (Q1) to third quartile (Q3), the line inside box represents median and minimum is calculated Q1-1.5*interqaurtile range (IQR) and maximum is calculated Q3+1.5*IQR, points outside the whiskers are outliers (Fig. 2). It shows that there are three outliers in test with reference 736A; these outliers were excluded from our dataset for further calculations (Table 1b). Because of non-normal

distribution of data with different number of values and repeating values, the exact Wilcox test was used. The p-values given by this test when testing all datasets with each other ranged between 0.18 to 1.00, which means the null hypothesis was accepted and antiviral activity of all samples was similar with significance level 5%.

The excellent antiviral treatment effect (*Mv* value above 3) was detected for all samples, concerning categories according to Annex F of ISO 18184 standard [9]. The values of antiviral activity of four tested samples are not significantly different (Figure 2 and Table 2a).

The results of the testing of 4 samples of treated textiles with confirmed antiviral activity have shown, that bacteriophage Phi6 is an optimal surrogate enveloped virus for the testing of antiviral activity of textiles as porous surfaces. Phi6 has many advantages for antiviral activity testing – resistance to environmental conditions, fast and safe processing, temperature optimum near room temperature [14]. The cultivation of bacterial host cells differs from the cultivation and preparation of animal cells as hosts of animal viruses. The advantages of the bacterial host cells are the fast growth, agar-based techniques of detection and the resistance against wide spectrum of environmental conditions. The benefit of bacteriophage-based method is the possibility of agar-diffusion preliminary test. It is allowed by completely different detection of infectious particles.

The results of the preliminary agar diffusion test (Table 3, Figure 3) have shown the antibacterial and antiviral activity of all tested samples, which confirms the leaching type of the antiviral treatment used for all 4 samples. Inhibition zones slightly differ depending on the textile material and their contact with agar surface. No inhibition was detected with the use of square samples of reference untreated material.

The antiviral substances of the un-leaching type are commonly preferred for the multiply-used products that must retain their antiviral properties over many washing cycles, but leaching type textiles may be more biocidal, on the other side. Specific technics, as dipping-padding-drying process or sol-gel process, help to prepare durable, non-leaching antipathogenic finishing of fabrics. The leaching-type textiles are commonly used for single-used textile products (surgical gowns, surgical caps) and specifically developed for dermatological applications (cotton wound dressing) [4] [17] [18]. The fast preliminary testing of antimicrobial/ antiviral activity and estimation of the stability of the antiviral treatment is the benefit of bacteriophage-based method of antiviral activity determination.

Table 2b. Values of antiviral activity of individual specimens determined with the use of 2 different reference materials signed 736A and 737A. Each test run was provided with 3 treated specimens and 3 untreated reference specimens. Outliers, identified by boxplot analysis, are marked by red colour. Values obtained by the inclusion of outliers are marked by blue colour. Marked values were excluded from dataset used for further calculations.

Reference				**736A**				
Sample	1	1	2	2	3	3	4	4
Mv1	4.36	4.36	4.36	4.36	3.66	4.36	4.36	4.36
Mv2	4.36	4.36	4.36	4.36	2.98	4.36	3.58	4.36
Mv3	3.41	4.36	4.36	2.88	4.36	4.06	4.36	4.36
SD	0.55	0	0	0.85	0.69	0.17	0.45	0
Mv average	4.04	4.36	4.36	3.87	3.67	4.26	4.10	4.36
Mv	4.20		4.12		3.97		4.23	

Reference				**737A**				
Sample	1	1	2	2	3	3	4	4
Mv1	4.52	3.97	4.52	3.97	3.82	3.97	4.52	3.97
Mv2	4.52	3.97	4.52	3.97	3.14	3.97	3.74	3.97
Mv3	3.57	3.97	4.52	2.49	4.52	3.67	4.52	3.97
SD	0.55	0	0	0.85	0.69	0.17	0.45	0
Mv average	4.20	3.97	4.52	3.48	3.83	3.87	4.26	3.97
Mv	4.09		4.00		3.85		4.12	

Table 3. Inhibition of growth of cells (*Pseudomonas* sp. DSM 21482) and plaque formation (bacteriophage Phi6 DSM 21518) in agar diffusion test.

	Inhibition of cells and virus in diffusion test			
Treated sample	Sample 1	Sample 2	Sample 3	Sample 4
Inhibition zone	++	++	+	++

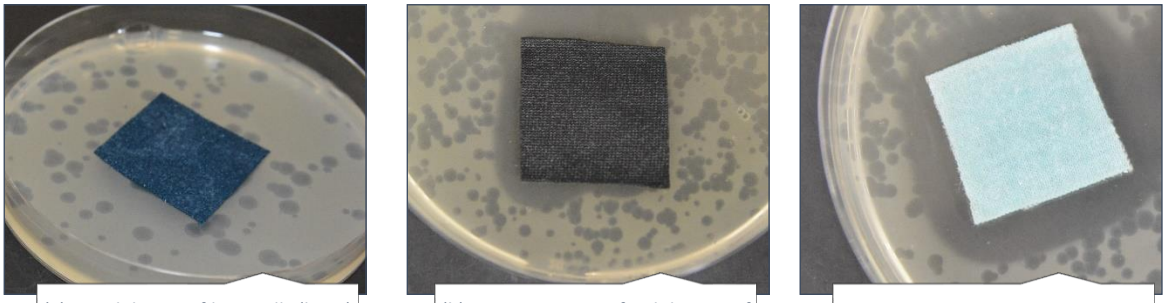

Figure 3. Agar diffusion test of textiles on double layer agar plate. (a) textile sample without antiviral treatment (knitted sample of blue colour), (b) sample 3 – narrow zone of inhibition of host strain and the bacteriophage phi6, (c) sample 4 – wide zone of inhibition of growth of cells and virus.

In this study, the method of antiviral activity testing of textiles was adapted to testing with a bacterial virus, enveloped bacteriophage phi6, surrogate of enveloped human viruses SARS-CoV-2, influenza virus and Ebola virus. The surrogate bacterial virus with different cultivation technique allows for efficient and fast testing without the influence of side effects of host-cell virus system (for example the need for medium dillution or the surface passivation of Ag nanoparticles, described by Imoto [19]). The described method is available for wide spectrum of testing purposes including the testing of antiviral activity of surfaces and textiles directly in the environment where they are used. Testing in real conditions can then follow up the verification of the antiviral effect of the tested material.

The bacteriophage-based method of antiviral activity testing allows to test antiviral properties of comprehensive range of samples in the frame of projects aimed on development of new antiviral textiles. The experimental design based on the comparison of specimens treated by different concentrations of active compound or produced by different finishing methods can work with a sufficient number of samples. Its advantage is rapid and accurate quantification (nearly all bacteriophage particles are infectious) via plaque forming units number determination.

CONCLUSIONS

The development and utilization of antiviral fabrics represent a significant advancement in the field of textile technology, with the potential to contribute to public health and safety. Innovations in the field of antiviral textiles, including biosensor materials, are not possible without effective and safe methods of antiviral activity testing. From this point of view, bacteriophages can be valuable tools in the evaluation of antiviral treatments or materials, providing comparable results for textile products evaluation. This paper describes the optimized method of antiviral efficiency testing available in conditions of BSL-2 laboratory equipped for common microbiological testing. The bacteriophage-based method allows to determine antiviral activity of wide spectrum of textile products, developed for the use in healthcare, public transportation, catering, space research. The effective testing method can help to develop new functional, health-friendly and environmental-friendly textile products.

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