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ORIGINAL ARTICLE

Antimicrobial activity assessment of textiles: standard methods comparison

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Abstract Antimicrobial fabrics are increasingly important in a great variety of applications and thus several standard methods to evaluate their efficiency have been developed. However, there is no consensus on the most adequate method to be used. Therefore, aim of this work was to compare the practical applicability of the best known standards: AATCC 147, ISO 20645:2004, AATCC:100 and JIS L 1902. Four samples, with different amounts of antimicrobial agents, were used. It was tested 3 qualitative methods (AATCC 147, ISO 20645 and JIS L 1902–Halo method) and 2 quantitative (AATCC 100 and JIS L 1902–Absorption method). For each method, both Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Klebsiella pneumoniae*) bacteria were used. Textiles samples assayed did not present diffusible activity, thus only the qualitative results from the AATCC 147 and the Halo method could be analyzed and no differences were observed between them. Therefore, the AATCC 147 or the JIS L 1902–Halo method can be used for a simple and expedite screening of a large amount of samples with or without diffusible antimicrobial activity. In contrast, the ISO 20645 can only be used when diffusible antimicrobial agents are present. Concerning the two quantitative methods, the results showed that the JIS L 1902 method is more sensitive to the amount of antimicrobial agent than the AATCC 100 test. An additional assay also showed that the JIS L 1902 is sensitive enough to distinguish serial dilutions of the antimicrobial agent.

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Introduction

Functional fabrics have become an important issue in the textile industry, especially the antimicrobial ones. Generally, fabrics provide a good contact area and can absorb moisture, both required for microbial growth. This growth can lead to malodours, dermal infections, allergic responses and fabric deterioration (Ammayappan and Moses 2009; Gao and Cranston 2008; Singh et al 2005). Thus, the incorporation of antimicrobial agents on textile products, able to overcome these problems, is of utmost importance.

Several standard methods to assess antimicrobial activity on textile product have been published. The most important standards include both qualitative: AATCC 147:2004, ISO 20645:2004 and JIS L 1902:2008–Halo method and quantitative methods: AATCC 100:2004, ISO 20743:2007–Absorption method and JIS L 1902:2008–Absorption method (Askew 2009).

The agar diffusion or qualitative methods are simple to perform, quick and useful when a large number of samples have to be screened. These methods consist of placing the textile samples in contact with nutrient agar (NA) plates containing bacterial cells (Gao and Cranston 2008). In the AATCC 147, samples are placed over NA layer, previously streaked with an inoculum of test bacteria (AATCC 147 2004). In the ISO 20645, fabrics are positioned between two-layer agar plates; the lower layer only with agar and the upper layer inoculated with selected bacteria (ISO 20645 2004), while in the JIS L 1902–Halo method samples are placed on only one agar layer containing cells (JIS L 1902 2008). These qualitative

methods evaluate the bacterial activity by the halo formation (absence of bacteria growth immediately around the edges of the fabrics). The halo size provides some indication of the potency of the antimicrobial activity of textile samples (Gao and Cranston 2008) but cannot be used as a quantification method.

Absorption or quantitative methods provide values of antimicrobial activity based on the reduction of planktonic bacterial growth (Askew 2009; Gao and Cranston 2008). However, they are more time and material consuming than the qualitative methods. In quantitative methods, a small volume of bacterial inoculum is placed in direct contact with the fabric samples, allowing the absorption of all liquid. After incubation, bacteria from the fabric are eluted and the total bacterial number is determined by serial dilution. Antimicrobial activity can be obtained as the percentage of reduction, determined against a control, without antimicrobial agent (Gao and Cranston 2008). Furthermore, there are only few differences between the methods, namely, bacteria incubation and elution process.

Although there are several standards, there are no practical studies, to the authors' knowledge, concerning the comparison of the available methods in order to access the most efficient. Hence, the main goal of this work was to make a practical comparison of both quantitative and qualitative methods described on the AATCC 100, AATCC 147, ISO 20645 and JIS L 1902, to determine the best method—more sensitive to the amount of antimicrobial agent on fabrics and with application on both diffusible and non diffusible antimicrobial agents..

Materials and methods

Microorganisms and samples

The microorganisms used in all assays were *Staphylococcus aureus* (ATCC 6538) and *Klebsiella pneumoniae* (ATCC 11296), selected according to the standards.

Four samples (with identical fabric composition) were tested: Sample A—positive control (with diffusion antimicrobial agent), Sample B—negative control (without antimicrobial agent), Sample C—with low amount of non-diffusion antimicrobial agent, and Sample D—with high concentration of non-diffusion antimicrobial agent. The antimicrobial agent present on the fabrics C and D was silver ions and on sample A was Tinosan.

For an extra assay, nine samples of another fabric were produced with serial dilutions of an antimicrobial agent.

Due to patent issues, the exact composition of the fabrics cannot be showed.

Qualitative methods

The qualitative determination of antimicrobial activity was made based on the protocol of the AATCC Test Method 147-2004, ISO 20645:2004 and the Halo method from JIS L 1902:2008.

In order to perform the AATCC 147 method, an inoculum was prepared as follows: 1.0±0.1 ml of a 24 h culture in nutrient broth (NB) was transferred into 9.0± 0.1 ml of sterile distilled water. With an inoculating loop, five streaks of the diluted inoculum were made over a standard Petri dish with nutrient agar (NA), without refilling the loop. The textile samples were placed over the streaks, ensuring intimate contact with the agar surface. The Petri dishes were incubated for 24 h at 37±2°C (AATCC 147 2004).

In the ISO 20645 method, the textile samples were placed between two agar layers. The lower layer contained 10±0.1 ml of NA and the upper layer had 5±0.1 ml of NA with 6.7×10^5 cells ml^{-1} of the bacteria. The bacteria came from a previous NB inoculum incubated for 24 h at 37±2°C (ISO 20645 2004).

The protocol from JIS L 1902–Halo Method was performed as follows. A previous NB inoculum was incubated for 24 h at 37±2°C. Then, 1.0±0.1 ml from the inoculum with 1×10^7 cells ml^{-1} was added to 15 ml of NA warmed at 45–46°C. This solution was disposed in a sterilized Petri dish. After agar solidification, the textiles samples were placed over the agar, and incubated for 24 h at 37±2°C (JIS L 1902 2008).

The evaluation of the antimicrobial activity, for all methods, was made based on the measure of the halo formed around the edges of the samples and the bacteria growth under the samples.

For the three methods, square fabric samples of 2×2 cm were used. Once the fabrics curl eedasily, it was necessary to use an acrylic coupon with the same size of the samples to flatten the samples. Tests were performed to ensure that the acrylic coupon had no antimicrobial activity. The bacteria concentration of the inoculum was achieved by the absorbance method, through the corresponding calibration curves.

Quantitative methods

The quantitative determination of antimicrobial activity was based on the Absorption method from JIS L 1902:2008 and AATCC Test Method 100-2004 protocols.

For the AATCC 100 method, a square sample of 4.8× 4.8 cm was used and, for the JIS L 1902–Absorption method, a sample of 0.4 g was used, according to the requirements of each standard.

The JIS L 1902–Absorption method was executed as follows: first, an inoculum was prepared in 20±0.1 ml of

NB and incubated for 24 h at 37±1°C. Then, bacteria concentration was adjusted to 3×10^8 cells ml^{-1}, by absorbance reading and using the respective calibration curves. A volume of 400 μl from the previous suspension was added to 20 ml of NB and incubated for 3 h at 37±1°C. The bacteria concentration was measured again and diluted in NB 20× (in distilled water) to 3×10^5 cells ml^{-1} and 200 μl of this inoculum were added to each sample. The samples were incubated for 24 h at 37±1°C. Then, 20 ml of physiological saline solution (8.5 g of NaCl and 2.0 g of non-ionic surfactant Tween 20 from Sigma per liter) was added to samples which were vortexed. In order to achieve the number of living bacteria, a serial dilution plate count method was performed (JIS L 1902 2008).

In the AATCC 100 method, an inoculum with 100 ml of NB and incubated for 24 h at 37±1°C was used. Its bacteria concentration was adjusted with NB to 2×10^5 cells ml^{-1}. Then, 1±0.1 ml of the diluted inoculum was placed in each sample. The samples were incubated for 24 h at 37±1°C. After the incubation period, 100 ml of physiological buffer solution (8.0 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4 and 0.2 g de KH_2PO_4 per liter of distilled water) was added and the samples were mixed in the vortex. In order to achieve the number of living bacteria, the serial dilution plate count method was performed (AATCC 100 2004).

All assays were performed in duplicate and repeated three times.

The percentage reduction of bacteria after 24 h incubation was calculated for both methods by the formula: [(no. bacteria on control fabric − no. bacteria on treated fabric)/ no. bacteria on control fabric] × 100.

Results

Various methods have been described to determine the efficacy of antimicrobial fabrics. Usually, these methods can be divided in two categories: qualitative and quantitative (Askew 2009; Gao and Cranston 2008).

Qualitative methods

In this work, three qualitative methods were used (AATCC 147, ISO 20645 and JIS L 1902–Halo method) to test the antimicrobial activity of samples. As was expected, the positive control presents a halo around the sample (Fig. 1a) and the negative control did not present any halo (Fig. 1b), for all three methods. It can also be observed that Samples C and D (Fig. 1c and d) did not present any halo either, indicating that these samples did not possess diffusible activity.

Although no halos were detected, after each assay, the samples used in tests AATCC 147 and JIS L 1902–Halo method were removed from the Petri dishes, thus allowing the analysis of microbial inhibition under the sample (Fig. 2). For the ISO 20645 method, it was impossible to remove the fabrics, because they were placed between two agar layers. From Fig. 2b, it is possible to see that sample B (negative control) did not repress the bacterial growth in contrast to Sample A (Fig. 2a). Sample D (Fig. 2d) inhibited *S. aureus* growth under itself for both methods. Sample C (Fig. 2c) did not showed inhibition of the bacterial growth for the JIS L 1902–Halo method; however, a small inhibition was observed when the AATCC 147 was used.

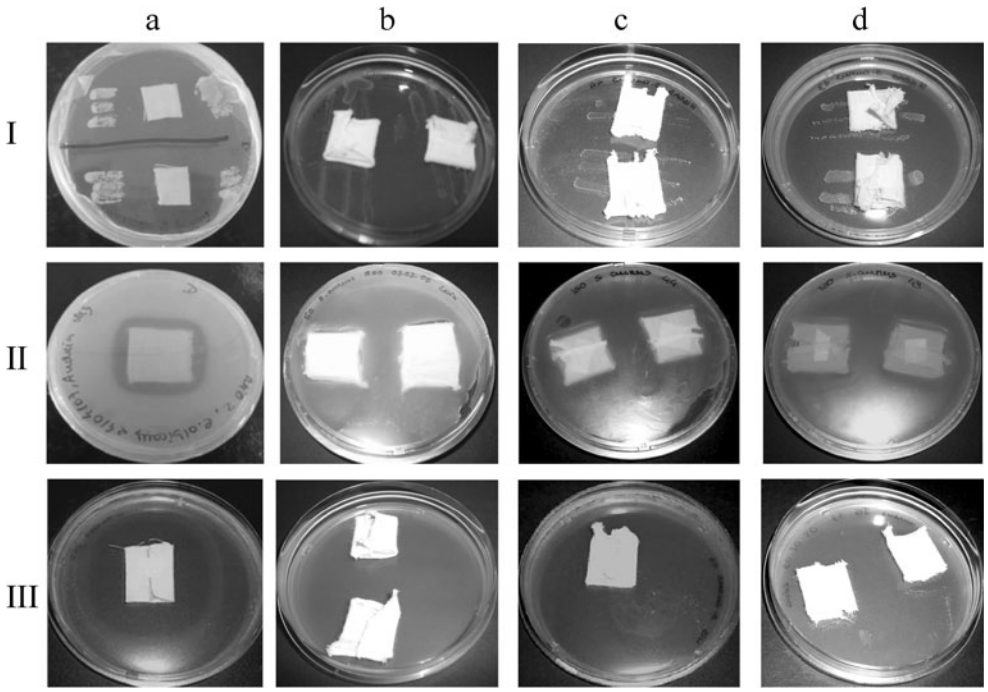

Fig. 1 Samples analysed with AATCC 147 (*I*), ISO 20645 (*II*), and JIS L 1902–Halo method (*III*). The assays were performed using *S. aureus*. **a** Positive control (Sample A), **b** negative control (Sample B), **c** Sample C, **d** Sample D

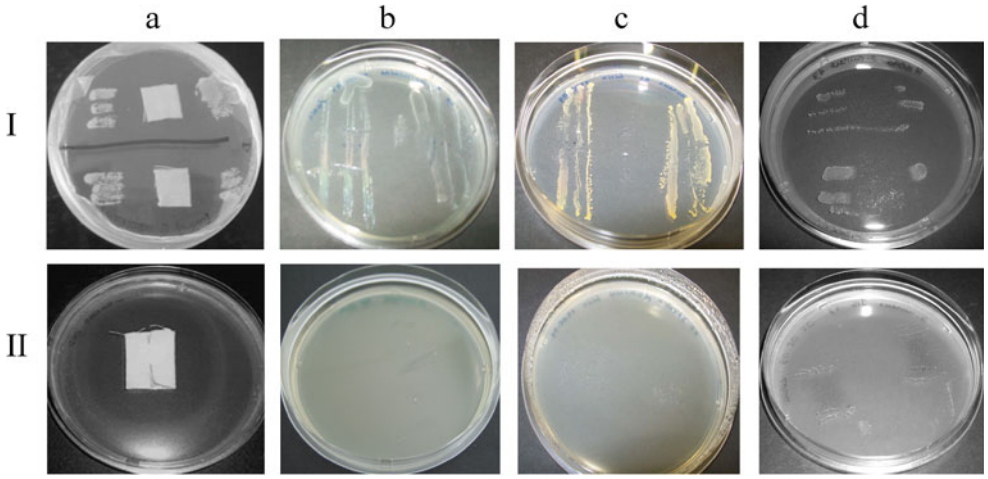

Fig. 2 Images of samples analysed with AATCC 147 (*I*) and JIS L 1902–Halo method (*II*) after fabric removal. The assays were performed using *S. aureus*. **a** Positive control (Sample A), **b** negative control (Sample B), **c** Sample C, **d** Sample D

Besides *S. aureus* (Gram-positive bacteria), and according to the standards, *K. pneumoniae* (Gram-negative bacteria) was also used. For the latter, similar results were observed for samples A, B and D. However, for sample C, results were different, once no growth inhibition under the fabric was observed when AATCC 147 was used (data not shown).

Quantitative methods

Besides qualitative methods, quantitative tests were also used. These methods allowed an accurate determination of the percentage of microbial growth inhibition. Each standard suggest the determination of specific parameters. Hence, in the present study, the percentage of growth inhibition was determined for both methods in order to allow the comparison between them.

The percentage of inhibition of both *S. aureus* and *K . pneumoniae* caused by samples A, C and D (determined using sample B as negative control), obtained by the JIS L 1902–Absorption method and the AATCC 100 method is presented in Fig. 3.

Regarding the results obtained with the JIS L 1902–Adsorption method, it is possible to notice (Fig. 3a) that samples A and D presented 100% of growth inhibition for both bacteria, while sample C presented only 35% for *S. aureus* and 100% of inhibition for *K. pneumoniae*. Similar results were achieved using the AATCC 100 method (Fig. 3b); however, sample C showed higher inhibition of *S. aureus* growth.

In addition, a supplementary assay, with the JIS L 1902–Adsorption method, was performed with textile samples made with increasing antimicrobial agent concentrations. Figure 4 shows a direct relation between the percentage of antimicrobial agent and the percentage of growth inhibition.

Discussion

Antimicrobial textiles can prevent fabric spoilage by microorganisms and can be used as medical devices, to prevent dermal infections or microbial growth, especially in hospitals (Askew 2009; Elsner 2006). Because of their growing industry, different methods to measure antimicrobial textile activity have been published in recent years.

The qualitative tests had filled a need for a relatively quick and easy way to determine antimicrobial activity on treated fabrics. In these tests, if a diffusible antimicrobial activity is present, a clear zone around the sample—a halo—will appear (Teufel and Redl 2006). In this work, three qualitative methods were analyzed. These standards assume

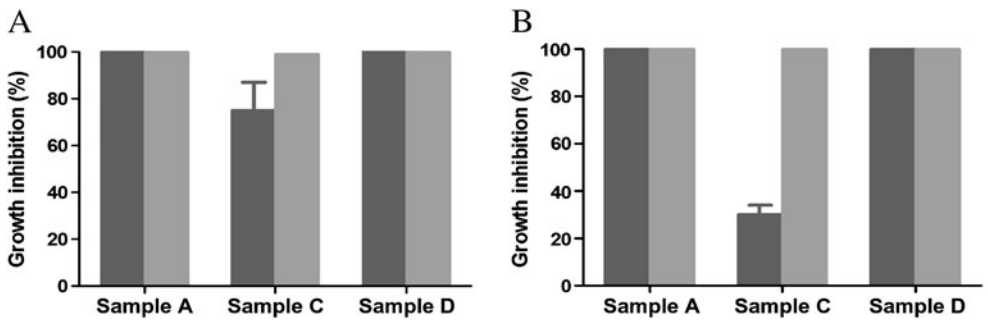

Fig. 3 Percentage of microbial growth inhibition obtained for JIS L 1902–Absorption method (**a**) and AATCC 100 method (**b**), testing Sample A, Sample C and D (negative control—sample B—was used to determine the percentage) against *S. aureus* (*dark gray*) and *K. pneumoniae* (*light gray*). *Bars* standard deviation

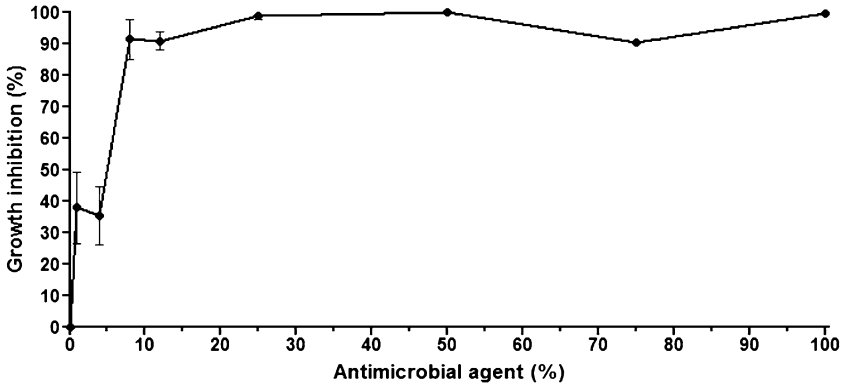

Fig. 4 Percentage of growth inhibition of *S. aureus* caused by textile samples with different percentages of antimicrobial agent for the JIS L 1902–Adsorption method

that a fabric has antimicrobial activity when a halo is formed (diffusible activity). However, the fabrics can have antimicrobial activity by direct contact, when there is no growth under the fabrics—no diffusible activity (Höfer 2006). AATCC 147 and JIS L 1902–Halo methods allow the removal of the sample, making possible the observation of bacterial growth inhibition under it. Moreover, it was confirmed in this work (Fig. 2) that inhibition under the fabric can be observed without any diffusible activity being displayed (Fig. 1). Although the methods were made for the assessment of antimicrobial activity of diffusible agents on textiles, it is possible to use them for the determination of the antimicrobial activity of non-diffusible agents. The only exception is the ISO 20645 method, because the samples were placed between two agar layers and so it was not possible to verify if there was any growth inhibition under the textile samples .Therefore, this drawback of ISO 20645 makes this test only applicable to antimicrobial textiles with diffusible activity, otherwise the results obtained can be misleading.

The quantitative methods, like the AATCC 100 and the JIS L 1902–Absorption methods, allow the determination of the exact value of the antimicrobial activity obtained by the calculation of the reduction of bacterial growth, against a control (Singh et al. 2005; Torlak 2008). Additionally, the ISO 20743–Absorption method is frequently used as a standard method; however, it was not considered in this study once the JIS L 1902–Absorption method was revised considering the harmonization with ISO 20743:2007.

However, the three standards suggest different parameters to access the activity of the antimicrobial textile. For instance, the AATCC 100 proposes the determination of percentage of bacteria reduction and the JIS L 1902/ISO 20743 suggests the calculation of the bacteriostatic and bactericidal activity. Besides, the results obtained with both methods were different: the determination of the antimicrobial activity with different formulas did not allow an immediate comparison of the results. Therefore, in this work, the parameter chosen to evaluate the methods' efficiency was the percentage reduction of bacteria (see formula in "Quantitative methods".

According to the results achieved with the quantitative methods (Fig. 3), samples A and D presented 100% of growth inhibition for both bacteria, confirming the high antimicrobial activity obtained in the qualitative methods (Fig. 2d). However, for sample C, the results were not consistent between the qualitative and quantitative methods, since, when assayed by the qualitative method, this sample did not present antimicrobial activity for either bacteria tested (*S. aureus* and *K. pneumoniae*) while the opposite was obtained for the quantitative methods (Fig. 3). Thus, this fact highlights the discrepancies and the misleading results that can be obtained with the standard methods available. Additionally, the lower inhibition of *S. aureus* is consistent with the sample lower content of antimicrobial agent.

The standard methods suggest the use of Gram-positive and Gram-negative bacteria species. This is required because the two types of bacteria differ in their cell wall and, consequently, on their susceptibility to antimicrobial agents. The Gram-positive bacteria have a continuous cell wall of a thick layer of peptidoglycan while Gram-negative bacteria have a non-continuous cell envelope formed by a thin layer of peptidoglycan covered by an outer membrane. Hence, Gram-negative bacteria are more susceptible to antimicrobial agents than Gram-positive ones (Baron 1996; Madigan et al 2000). As far as sample D is concerned, the qualitative methods did not display differences in the antimicrobial capacity of the fabrics against both bacteria. However, when assayed by both quantitative methods, sample C showed lower inhibition for *S. aureus* than for *K. pneumoniae*, as should be expected due to their cell wall structure. The differences of antimicrobial activity found against the two bacteria highlights the importance of including both types of microorganisms when performing these tests.

Furthermore, an additional assay was performed with samples having increasing percentages of antimicrobial agent. The method selected was the JIS L 1902–Adsorption method, once it was shown to be the most accurate. It could

be observed (Fig. 4) that the method is sensitive enough to distinguish samples with similar amounts of antimicrobial agent, allowing the establishment of a relationship between the percentage of antimicrobial agent and the effect on bacterial growth. Moreover, it will enable the determination of the optimum percentage of antimicrobial agent to be used. Therefore, this result highlights and confirms the accuracy of the JIS L 1902–Adsorption method.

This comparative study of the available standards for textile antimicrobial activity assessment clearly showed that the quantitative tests (AATCC 100 and the JIS L 1902–Absorption method) are more accurate and reliable than the qualitative ones. It was also possible to verify that JIS L 1902–Absorption method is very sensitive.

Although the qualitative methods (AATCC 147 or the JIS L 1902–Halo methods) were not the most profitable, they can be used for the first screening of a large number of samples. It should be pointed out that, although these two methods were prepared for diffusible antimicrobial agents, it was possible to use them on non-diffusible agents.

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