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Method for estimating bioburden of nonsterile cotton gauze

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In new hygienically controlled plants, products often have a low level of microbial contamination, so that current methods for estimating bioburden appear to be inadequate. The adoption of efficient procedures giving consistent and reproducible results could contribute to the improvement of conventional methods for evaluating microbiological quality of products with low bioburden. The effectiveness of a washing procedure and mechanical shaking for the removal of *Bacillus subtilis* spores from pre-inoculated cotton gauze samples was tested in combination with a membrane filtration technique. A 45-min agitation in the presence of surfactant and glass beads improved recovery up to 70.5%, with satisfactory reproducibility. In order to compare the procedure with the current standard method, uncontaminated samples were processed to extinction by applying a repetitive treatment. When exhaustive rinses were performed in order to calculate a conversion factor, permanent entrapment of a high percentage of organisms in the cotton microfibers was highlighted: this fact may play a role in an overestimation of the extrapolated removal efficiency.

Keywords: bioburden; recovery; cotton gauze; spores removal

Introduction

Microbiological control in gauze manufacturing plants is generally assessed by sterility testing of products that have been subjected to ethylene oxide sterilization. For a new hygienically controlled plant we were requested to set up a simple and manageable method both to evaluate microbial contamination on products which are supplied nonsterile but for which microbiological cleanliness is specified, and to monitor the contamination level of materials throughout the manufacturing environment (storage, machine processing and packaging).

Standard methods for bioburden assessment, published both by AAMI [1] and by CEN [4] recommend establishing the recovery efficiency by employing naturally contaminated samples and recovering to extinction by repetitive treatment (exhaustive rinse). The use of clean rooms has so markedly reduced the number of microbial contaminants on the products that preliminary experiments indicated a need for sampling a larger amount of cotton gauze to assure consistent numbers of microorganisms.

This report describes the development of a method for determining, by employing pre-inoculated samples, an estimate of the number of viable organisms (bioburden) on cotton gauze compared with the standard method applied to uncontaminated samples.

Materials and methods

All work was carried out in a vertical air-flow cabinet (Twin 30, ICN, Biomedical Inc, Costa Mesa, CA, USA). Each sample of gauze was weighed (2 g), aseptically cut into 2-cm^2 squares and, with the aid of sterile forceps, placed in a sterile 1-L bottle containing 200 ml of sterile normal saline, with 0.01% Tween 80 as a dispersant. The bottom of the flask was covered with glass beads, the bottle was screw-capped and placed horizontally in a reciprocating shaker (Kahn shaker, TKA, Teknolabo, Milan, Italy) and shaken at 270 oscillations per minute, for 45 min.

After the shaking procedure, 100-ml samples of the eluent were individually filtered through membrane filters (Sartorius CN, pore size 0.45 μm, Sartorius AG, Goettingen, Germany). The filtration equipment was a Dasit 3-place manifold type (German Science Int, Ann Arbor, MI, USA) holding polysulfone autoclavable magnetic funnels. The eluent was decanted slowly so that no glass beads or gauze fragments fell through to the filter. The membrane was then rinsed twice with 50 ml of sterile normal saline to remove Tween 80 and foam traces. After filtration, the membrane was transferred by sterile membrane forceps to a 50-mm Petri plate of Tryptic Soy Agar (TSA, Difco Laboratories, Detroit, MI, USA). Colony growth was examined daily and colonies were counted under a magnifying glass, after 24 and 48 h of aerobic incubation at 32°C.

To achieve a useful quantitative parameter of recovery efficiency, we selected spores of *Bacillus subtilis* var *niger* (ATCC 9372) (Difco). The suspension was adjusted to give approximately 1.5×10^3 CFU ml^{-1} and 0.1 ml was applied to the surface of samples (1 g) of sterile gauze, allowed to dry under laminar air flow and stored overnight at 4°C. Inoculum levels were determined by plating 0.1 ml of the suspension on TSA plates (average of 10 plates). The technique was employed to establish the number of inoculated microorganisms which are removed from the product and the removal efficiency was determined as the ratio between the inoculated level and the amount of recovery, in order to provide a correction factor applicable in estimating the

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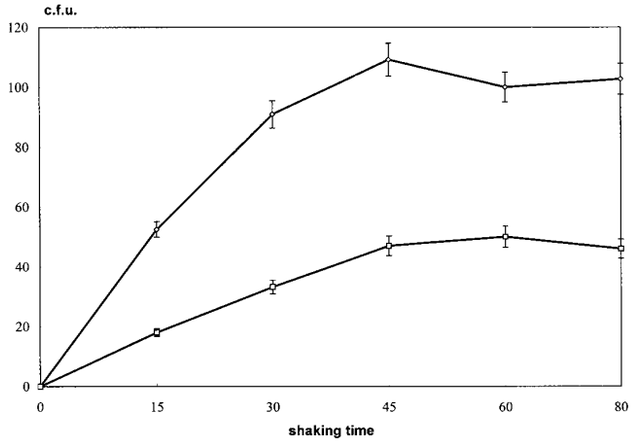

Figure 1 Recoveries of spores from cotton gauze using different treatments. Treatment 1, —◇—, eluent is sterile normal saline + 0.01% Tween 80 + glass beads. Treatment 2, —□—, eluent is sterile normal saline only. The gauze sample (2 g) was suspended in 200 ml washing fluid.

actual bioburden. A number of trials were performed by shaking inoculated samples for various times from 15 to 80 min, with and without Tween 80 and glass beads.

In order to compare the procedure with the standard bioburden method recommended by both AAMI [1] and CEN [4], samples with a natural contamination were assayed. When uncontaminated gauze samples were preliminarily assayed, the total number of colonies per plate was too low for satisfactory calculation, indicating a need for sampling larger volumes of wash solution.

Each plate was accordingly inoculated with the material obtained after filtering 4×200 ml of eluent (originating from 8 g of cotton gauze. We recovered to extinction, reprocessing the samples by a repetitive treatment. Counts were determined for each cycle, and finally the exhausted products were coated with melted TSA medium, it was allowed to solidify and the plates were incubated as above; the colonies formed were counted. The number of colonies counted after the initial application of the removal technique was expressed as a fraction of the total number of colonies detected, and used to establish the removal efficiency by calculating a conversion factor.

Results

Comparisons made between agitation with and without glass beads + Tween 80 showed that significantly more spores were recovered from samples of cotton gauze in the former treatment. Table 1 summarizes the comparative efficiency obtained with the two treatments at different experimental times.

In absolute numbers, more colony forming units (CFU) were obtained at 45 min of shaking in the presence of Tween 80 + glass beads and percent recovery was significantly higher; longer shaking times did not improve microbial removal. Table 2 shows the results of the samples with a natural bioburden processed by the standard method: counts related to the exhaustive rinses are reported. Lower recoveries were obtained on cycle 1, and a higher percent of microorganisms appeared to be permanently entrapped between the microfibers when normal saline alone was employed.

Discussion

The results suggested that recovery rate in cotton gauze is affected by the washing procedure: shaking time appears critical. A vigorous shaking, the use of glass beads to increase surface abrasion and, possibly, the surface active properties of the eluent contribute to recovery efficiency. These results are in accord with those published on a different material, Latex surgical gloves [6].

Maximum release of spores is delayed compared to agitation times as recommended on medical devices (15–20 min) [3] and as applied to a number of smooth materials: filter paper (15 s) [2], glass and polymers (5 min) [5], steel and glass (20 min) [7]. The microstructure nappiness of cotton presumably interferes with the ready liberation of spores; results suggest adhesion or entrapment between cotton microfibers.

It is evident that higher estimations can result by applying the correction factors reported in Table 1 to the counts of the uncontaminated samples (Table 2) after cycle 1 (30 min) and cycles 1 + 2 (60 min). The gap between the results of the two methods can perhaps be attributed to the fact that organisms trapped in the weft threads of the exhausted cotton gauze escape detection. The practical risk of overestimating the removal efficiency by applying the standard

Table 1 Recoveries and calculated correction factors by treatment with and without Tween 80 + glass beads

Shaking time (min)	Sterile normal saline + 0.01% Tween 80 + glass beads			Sterile normal saline		
	Colony counts CFU ± s.d.	Recovery[a] %	Correction factor	Colony counts CFU ± s.d.	Recovery[a] %	Correction factor
15	52.5 ± 1.9[b]	33.9	3.0	18.0 ± 4.0	11.6	8.6
30	91.0 ± 3.3	58.7	1.7	33.2 ± 4.0	21.4	4.7
45	109.2 ± 3.0	70.5	1.4	47.0 ± 5.2	30.3	3.3
60	100.0 ± 3.1	64.5	1.6	50.0 ± 4.1	32.3	3.1
80	102.7 ± 3.1	66.2	1.5	46.0 ± 5.6	29.7	3.4

[a]Inoculum level, plated on TSA, was 155.0 ± 3.0 (CFU ± s.d., avg of 10 plates).
[b]Each value represents the average of six determinations.

Table 2 Recoveries of samples with a natural contamination by a repetitive treatment with and without Tween 80 + glass beads

Cycle No. (30 min each)	Sterile normal saline + 0.01% Tween 80 + glass beads		Sterile normal saline	
	Colony counts[a] CFU ± s.d.	Recovery %	Colony counts[a] CFU ± s.d.	Recovery %
1	62.0 ± 6.6	80.6	30.0 ± 8.8	49.7
2	8.4 ± 3.8	10.9	7.4 ± 4.2	12.3
3	0.6 ± 0.9	0.8	1.0 ± 1.7	1.7
4	0.4 ± 0.6	0.5	0.6 ± 0.9	1.0
Agar overlay	5.6 ± 2.3	7.3	21.4 ± 9.0	35.7
Total colonies detected[c]	77.0 ± 14.1	100.0	60.4 ± 24.6	100.0

[a]Each number is the average ± s.d. of five plates, each inoculated with the material from 800 ml of wash solution from four 2-g samples of cotton gauze with a natural contamination.
[b]Each value represents the average of six determinations.
[c]Total counts were calculated by addition of the averages ± s.d. of each fraction.

method to cotton gauze, and presumably to other loosely-woven textile materials, has to be stressed.

Furthermore, spores of *Bacillus subtilis* have been found convenient because this model may mimic the contaminants of cotton gauze. Actually, because of the lack of essential nutrients, the dryness of material as well as some steps of the manufacturing process (scouring by NaOH, bleaching by H_2O_2 and air drying) at high temperatures and pressures, we could assume that the major contaminants of cotton gauze are spores.

It is justifiable to expect a low microbial content in non-sterile medical products; with the adoption of 'clean rooms' for the processing and packaging of nonsterile products, a new standard of cleanliness has evolved in medical device manufacturing plants, with a reduced number and type of contaminants accumulating on product surfaces. Because of the low level of microbial contamination, the current method for estimating bioburden is inadequate. The procedure reported here is efficient in terms of both labor and cost, providing a simple method for removing micro-organisms. It offers the advantage of more reliability in assuring proper evaluation of contamination of nonsterile cotton gauze and it also provides a suitable technique for routinely monitoring hygienic conditions throughout the entire manufacturing process of nonsterile products.

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