HOMOGENEITY AND STABILITY OF SAMPLES USED FOR PROFICIENCY TESTING IN ENUMERATION OF COAGULASE POSITIVE STAPHYLOCOCCI

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Abstract

This study describes preparation of test samples composed of freeze-dried strain of S. aureus and powdered milk as a matrix. In the first part of the study, the number of S. aureus cells freeze-dried in skim milk or horse serum were compared at two levels of contamination (10^4 and 10^5 cfu g^-1). The analysis of the samples was performed three times within a week. The preliminary results showed that the samples composed of S. aureus freeze-dried in horse serum were more stable and homogeneous than those prepared with skim milk. These results were further confirmed after analysing a higher number of such samples. Therefore, this procedure was then chosen for preparation of the samples for proficiency tests (PTs). Homogeneity and stability of these samples were checked according to ISO 13528. The results obtained showed that the samples met the criteria of stability and homogeneity required for PTs and were used in PT for enumeration of S. aureus in powdered milk.

Key words: S. aureus, homogeneity, stability, proficiency testing.

Material and Methods

Preparation of test samples. The PT included samples consisting of powdered milk as a matrix and freeze-dried test strain of Staphylococcus aureus ATCC 25923. To assess the optimal way of freeze-drying the bacteria, two series of experiments were performed at two levels of contamination: 10^4 cfu g^-1 and 10^5 cfu g^-1 in skim milk and horse serum, respectively. After choosing the best procedure of bacterial preparation, the final homogeneity and stability of test samples were performed in the frame of PT. S. aureus was cultured on brain heart infusion (BHI) broth (Oxoid, UK) at 37°C for 24 h. The bacterial culture was then mixed with skim milk or horse serum to obtain the final concentration of 10^4 cfu g^-1 or 10^5 cfu g^-1 in powdered milk. The mixtures were then frozen at -70°C before lyophilisation. The freeze-dried bacteria were stored at 2–8°C. The samples for the analysis were prepared by mixing the lyophilised bacteria with 10 g of milk powder. After the diluent (potassium dihydrogen phosphate) was added, the samples were incubated at room temperature for 1 h and analysed according to the EN ISO 6888-2 standard (1).

Scheme of the experiment. In the preliminary experiment, powdered milk mixed with freeze-dried S. aureus suspended in horse serum was used. The samples were analysed 3 times a week – immediately after freeze-drying (D0), after 4 d (D4), and after 7 d (D7),...
respectively. Simultaneously, at \(D_0\), the samples consisting of freeze-dried \textit{S. aureus} suspended in skim milk and powdered milk were also tested.

Considering the results of the analysis described above, the second part of the experiment was performed only with the samples fortified with \textit{S. aureus} strain freeze-dried in horse serum. Finally, the test samples for PT participants were prepared and their homogeneity and stability were checked according to ISO 13528 (4).

**Results**

During the preliminary experiments, it was found that powder milk samples mixed with \textit{S. aureus} freeze-dried in skim milk had a very low homogeneity (Table 1). For this reason, these samples were not included in the further tests. On the other hand, the samples with \textit{S. aureus} lyophilised in horse serum had a very low standard deviation, which indicated their good homogeneity. Furthermore, the difference between mean results of these samples obtained within 7 d was 0.06 for samples on low level of contamination and 0.01 for highly contaminated samples, which indicated also their good stability (Table 1). The preliminary results were further confirmed when a higher number of samples containing \textit{S. aureus} freeze-dried in horse serum were tested for their homogeneity. The results of these experiments are summarised in Table 2. It was noted that the number of bacterial cells was very stable when tested at days 0 and 4 and the difference between the mean results was 0.01 for \(10^5\) cfu g\(^{-1}\) and 0.08 for \(10^5\) cfu g\(^{-1}\).

The final experiment was then performed with the samples prepared for PT, which contained \textit{S. aureus} lyophilised with horse serum. The samples (two at each contamination level) were tested on a day of shipment to the participants. The homogeneity was investigated according to Annex B of ISO 13528:2005 where standard deviation between samples \((s_x)\) was compared with standard deviation for the proficiency assessment \((\sigma)\). The samples were homogeneous when \(s_x \leq 0.3\sigma\). The \(\sigma\) value calculated from the participant results was 0.08 and 0.10 at low and high levels of contamination, respectively. Thus, 0.3\(\sigma\) was calculated as 0.02 and 0.03, respectively, so the homogeneity criterion was met and the test samples were considered as homogeneous.

The stability test was also performed according to Annex B of ISO 13528:2005 and comprised the comparison of the average of the results obtained in the homogeneity check \((x_{.,.})\) with that of the results obtained in stability check \((y_{.,.})\). These samples in duplicate were analysed twice - before sending to the participants and then on the day of the analysis. It was found that the samples contaminated with a higher number of \textit{S. aureus} cells \((10^5)\) did not meet the criterion of stability \(|x_{.,.}−y_{.,.}| \leq 0.3\sigma\) but met the criterion calculated from the mean results between the first \((D_1)\) and the last day \((D_7)\) of analysis \(|x_{D_1}−x_{D_7}| \leq 0.2\log_{10}\) taken from the precision of the ISO 6888-2 method. The results of these studies are shown in Tables 3 and 4.

<table>
<thead>
<tr>
<th>Time of analysis</th>
<th>Level of contamination</th>
<th>10(^5) cfu g(^{-1})</th>
<th>10(^6) cfu g(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(skim milk (n=3))</td>
<td>horse serum (n=3)</td>
<td>skim milk (n=3)</td>
</tr>
<tr>
<td>(D_0)</td>
<td>Mean (log(_{10})/SD)</td>
<td>Mean (log(_{10})/SD)</td>
<td>Mean (log(_{10})/SD)</td>
</tr>
<tr>
<td></td>
<td>3.79/0.28</td>
<td>3.98/0.03</td>
<td>4.75/0.16</td>
</tr>
<tr>
<td>(D_4)</td>
<td>NA</td>
<td>3.95/0.04</td>
<td>NA</td>
</tr>
<tr>
<td>(D_7)</td>
<td>NA</td>
<td>4.04/0.08</td>
<td>NA</td>
</tr>
</tbody>
</table>

NA - not analysed, SD - standard deviation, \(D_0\) - first day, \(D_4\) - fourth day, \(D_7\) - seventh day

<table>
<thead>
<tr>
<th>Time of analysis</th>
<th>Level of contamination</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>10(^5) cfu g(^{-1}) (n=10)</td>
</tr>
<tr>
<td>(D_0)</td>
<td>Mean (log(_{10})/SD)</td>
</tr>
<tr>
<td></td>
<td>3.86/0.04</td>
</tr>
<tr>
<td>(D_4)</td>
<td>3.87/0.03</td>
</tr>
</tbody>
</table>

Symbols as in Table 1.
**Table 3**

| Level of contamination (cfu g⁻¹) | Mean (log₁₀) | SD | Homogeneity  
|---------------------------------|-------------|----|----------------|
| 10⁵ (n=20)                      | 3.72        | 0.02 | sₓ ≤ 0.3σ  
| 10¹ (n=20)                      | 4.68        | 0.06 | Σ  
|                                |             | 0.01 | 0.08  
|                                |             | 0.02 | 0.10  
|                                |             | 0.02 | 0.03  

SD - standard deviation, sₓ - standard deviation between samples, σ - standard deviation for proficiency assessment

**Table 4**

| Level of contamination (CFU g⁻¹) | Stability of the samples within 5 d | Stability  
|---------------------------------|-----------------------------------|----------------|
|                                 | Mean from D₀ analyse (log₁₀)/SD  | Mean from D₅ analyse (log₁₀)/SD  | Range between mean from D₀ and D₅  | xₜ, yₜ  | | xₜ, yₜ, | ≤ 0.3σ  
| 10⁴ (n=6)                       | 3.80/0.03                         | 3.71/0.02                         | 0.09                         | 3.720 | 3.710 | 0.01  
| 10⁵ (n=6)                       | 4.83/0.04                         | 4.75/0.03                         | 0.08                         | 4.682 | 4.754 | 0.07  

SD - standard deviation, xₜ - average of the results obtained in the homogeneity check, yₜ - average of the results obtained in stability check, D₀ - first day, D₅ - fifth day

**Discussion**

This study shows that using freeze-drying of *S. aureus* strains is a good way to obtain stable and homogeneous samples proficiency testing. However, *S. aureus* strains were stable when they were suspended in horse serum but not in skim milk before freeze-drying.

Grimaldi et al. (2) described the methods of setting up proficiency testing schemes for enumeration of *S. aureus*. In their studies, pure cultures of *S. aureus, Salmonella Typhimurium, and Escherichia coli* at various concentrations were mixed together with milk and 2 mL of the suspension was lyophilised. To obtain a test sample, the content of each vial was suspended in 2 mL of tryptone solution, and then divided into two portions of 1 mL each. Ten samples in duplicates were analysed to check homogeneity and five samples in duplicate were analysed to check stability of the test samples according to the instructions from “Harmonised Protocol for the Proficiency Testing” (8). The data obtained showed no suspect features including discordant duplicate results, outlying samples, trends, discontinuities or other systematic effects. Their observations, similarly to those obtained in the present study, indicate that freeze-drying is a useful tool to obtain homogeneous samples but the level of bacteria decreases after this process (for *S. aureus* from 10⁵ to 10³ cfu g⁻¹). However, our studies revealed that strains suspended in milk were not homogeneous. In the studies described by Peterz and Steneryd (7), selected cultures of bacteria were mixed with inositol serum broth, and 0.5 mL portions were frozen and lyophilised. The freeze-dried material was reconstituted in 54 mL of 0.1% peptone saline and then examined. The stability of the samples was checked by analysing three or five replicate vials every fourth week during a one year period. The homogeneity was checked by examination of five randomly selected vials. According to the authors’ observations, the number of the most microorganisms examined remained fairly stable. Additionally, the authors suggested that freeze-dried mixed cultures can be used to test performance of media and the reproducibility of testing methods in individual laboratories in qualitative and quantitative microbiological examinations. The stability and homogeneity of the samples were assessed during 1 year when stored at 2⁰C to 6⁰C. In our studies, similar temperature of storing of freeze-dried strains was used (2–8⁰C) but homogeneity and stability was performed only for one week. Considering the data reported by Peterz and Steneryd (7), it may suggest that samples prepared by us may remain stable and homogeneous also for a longer period of time and this will be tested in the future.

Before preparing test samples in the frame of PT, it is important to select the procedure of sample preparation that will guarantee their homogeneity and stability during participating in the PT. This choice depends on the organiser experience or from the results of experiments designed in the frame of preparation to organising the PT.
References


