COMPARISON OF THE SPREAD PLATE TECHNIQUE AND THE MPN-TECHNIQUE FOR THE DETERMINATION OF VIABLE COLONY COUNTS IN QUANTITATIVE SUSPENSION TESTS TO EVALUATE BACTERICIDAL AND FUNGICIDAL ACTIVITY OF CHEMICAL DISINFECTANTS

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Introduction

The European standards EN 1656[1] and EN 1657[2] specify suspension tests to establish whether a chemical disinfectant for use in the veterinary field has a bactericidal or fungicidal activity. The documents were prepared by the Technical Committee TC 216 "Chemical Disinfectant and Antiseptic" of the European Committee for Standardisation (Comité Européen de Normalisation, CEN) which was formed 1990 to harmonize and standardize the "effectiveness methods". On the basis of these suspension tests the results of the viable colony counts with the Spread Plate technique and the MPN-Technique (Most probable number) were compared. Incomplete neutralization of the bactericidal or fungicidal properties of the disinfectant may occur using the Spread Plate method. The MPN-technique is a method for quantitative determination of the viable colony count in a liquid medium, wherein a neutralizer is added to each tube of the dilution steps.

Based on these two counting methods the following questions should be answered:

- Are there significant differences between the results of Spread Plate and MPN technique?
- Is it possible to apply the MPN method to quantitative determination of fungi?
- Is it possible to use the MPN method as a replacement for the Spread Plate method?

Methods

In this study, the chemical disinfectant effectiveness was tested based on the suspension tests EN 1656 and EN 1657. This laboratory test took into account practical conditions of application of the product, including contact time, temperature, test micro-organisms and interfering substances.

The principle of the suspension test method (given in figure 1) was to dilute a sample of the product with water of standardized hardness and then add it to a mixture of test

suspension of bacteria or fungi and interfering substance. The "high level soiling" given in the EN standards was chosen as interfering substance with the final concentration of 10.0 g/l yeast extract and 10.0 g/l bovine albumin in the test procedure. The mixture was maintained at 10°C ± 1°C for the chosen contact times of 15, 30 and 60 min ± 10 s. At the end of the contact times, an aliquot was taken, and the bactericidal or fungicidal activity in this portion was immediately neutralized or suppressed by a validated method. The method of choice was dilution-neutralization. After a neutralization time of 5 min ± 10 s, a sample of 1.0 ml of the neutralized test mixture containing neutralizer, product test solution, interfering substance and test suspension was immediately taken in duplicate and diluted with diluent to 10^{-7} dilution. Out of each dilution step the Spread Plate method and the MPN method were inoculated in parallel. After incubation the numbers of surviving bacteria or fungi in each sample were determined and reduction was calculated.

For determination of the number of surviving test organisms in the MPN method the pattern of positive and negative tubes was noted each day, and standardized MPN table[3] was consulted to determine the most probable number of organisms per unit volume of the original sample.

For calculation of the reduction for each test organism the number of cfu/ml in the bacterial or fungal test suspension and of the results of the test was recorded and the decimal log reduction was calculated.

The trials based on EN 1656 were performed 10 times using *Staphylococcus aureus* as the test organism and formic acid and a commercial disinfectant preparation as active compounds.

In the trials according EN 1657 *Aspergillus niger* was used as the test organism. Formaldehyde and a commercial disinfectant preparation were used as disinfectants.

The product had passed the test when it demonstrated at least a 5 decimal log (lg) reduction (EN 1656) or 4 decimal log (lg) reduction (EN 1657) when diluted with hard water under simulated high level soiling (10 g/l bovine albumin solution plus 10 g/l yeast extract) under the chosen test conditions.

The data obtained from the test processed statistically using Microsoft Excel and SPSS programs to calculate the mean, minimum, maximum, median, variance and standard deviation. The significant difference between the two counting methods was derived using the so called Student's t-Test.

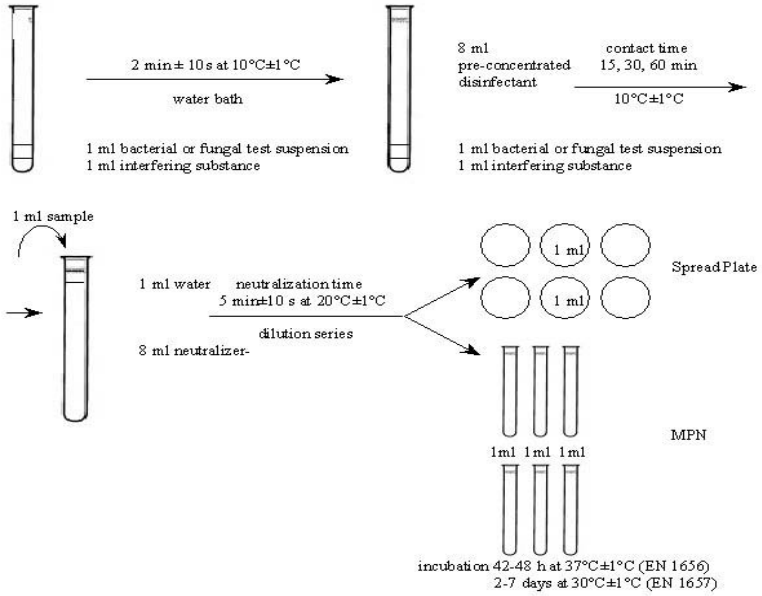

Figure 1: Quantitative suspension test (EN 1656, EN 1657)

Results

Using the Spread Plate technique counting of the colonies especially in the trials with *Aspergillus niger* after incubation was difficult due to the increasing number of colonies and the diameter size of the colonies. The plates became overgrown and showed no longer well-separated colonies.

The MPN method gave a better alternative for counting the colonies because the broth tubes were only observed for the presence or absence of growth. This method gives more benefit in colonies counting because the broth can be incubated for a longer time and still allows an accurate determination of the colony count.

In the trials based on EN 1656 using *Staphylococcus aureus* the MPN-technique tended to result in higher colony counts and consequently lower reductions after exposure to the disinfectant. However, significant differences were not seen at all tested disinfectant concentrations and contact times. The statistical spread of the single test results tended to be higher in the Spread Plate method.

Comparison of the results between Spread Plate method and MPN method in the trials with EN 1657 using *Aspergillus niger* did not show significant differences ($\alpha \leq 0.05$) in the colony counts of the fungi although the Spread Plate method gave higher reductions than the MPN method. However, significant differences between both methods were seen at the active concentrations of the tested disinfectants.

Discussion

During the effectiveness test, the SP method required more attention of time and precision. Result interpretation of the Spread Plate method took more time and effort than the MPN method. The reason was that in the Spread Plate method each plate had to be counted. The interpretation of the MPN method was easier, the pattern of growth was observed visually and then this pattern was compared with standardized MPN table. It means the MPN method offered an economic way from time and effort.

During the observation of growth result, in general the MPN method gave a higher colony count (cfu/ml) and in the consequence lower reductions compared to the Spread Plate method. The reason why this phenomenon could happen is that probably damaged cells can recover and then grow in the liquid medium, but not on the solid one due to the fact that in the MPN method the neutralizer was added to each broth tube while there was no neutralizer added to the agar medium used for Spread Plate technique. So transferred residual of the disinfectant was neutralized over a longer neutralization time in the MPN method and the subletal damaged test organisms had a chance for being repaired and showed growth.

MPN method also offers a probability to minimize the broth medium volume required by using a mini titter method which basically is a mini form of a normal MPN method which was used in this thesis. The advantages of this technique are a reduction of working time and material as a rising of trial quantity and reduction of substance doses needed for testing.

Conclusions

The MPN method is suitable to replace the Spread Plate method in EN 1656 and 1657 because it yields comparable results, it is less labour intensive, filamentous fungi can be determined quantitatively and the MPN-technique offers miniaturisation possibility. Further studies should determine whether the tendency of higher cfu/ml (lower reduction) in the MPN-method can be verified for other disinfectant products too.

References

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