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Methods for determining the presence of the foulbrood bacterium *Bacillus larvae* in honey

Metoder til påvisning af bipestbakterien Bacillus larvae *i honning*

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Summary

Two methods are described for establishing the presence of the foulbrood bacterium *Bacillus larvae* in honey. Both methods involve cultivation from honey on artificial media. There is no significant difference between the two methods. The presence of the bacterium can be determined when more than 10,000 spores per 5 g honey are found. Each colony which arises represents 5,000–15,000 bacterium spores.

Key words: *Bacillus larvae,* American foulbrood, honey, honey bees.

Resumé

I beretningen er der beskrevet to metoder til påvisning af bipestbakterien *Bacillus larvae* i honning. Ved begge metoder bliver der dyrket fra honningerne på kunstige substrater. Der er ikke signifikant forskel på metoderne. Bakterien kan påvises, når der er mere end 10.000 sporer pr. 5 g honning. Hver koloni, som genfindes, repræsenterer 5.000–15.000 bakteriesporer.

Nøgleord: *Bacillus larvae,* ondartet bipest, honning, honningbier.

Introduction

American foulbrood is a disease affecting the brood of honey bees. It is caused by the spore-forming bacterium *Bacillus larvae,* and bee colonies affected by the disease normally die after a few years if they are not treated. American foulbrood can therefore give rise to substantial losses both for beekeepers and seed producers. In Denmark and many other countries, the disease is treated by public authorities. The disease does not attack human beings.

There is longstanding evidence that American foulbrood can be spread by the honey of infected bee colonies. Accordingly, Danish provisions for the treatment of American foulbrood contain specific regulations concerning the honey of infected bee colonies (*Landbrugsministeriet,* 1976). As regards the provisions for the importing of honey, the State Inspectorate for Dairy Produce and Eggs is empowered to take samples of honey for analysis with a view to checking for bee disease (*Landbrugsministeriet,* 1978).

Sturtevant (1932, 1936) has provided a qualitative and a quantitative method for investigating honey for spores of *B. larvae*. Both methods have been tried out experimentally by the State Bee Disease Committee. The experience with these methods, however, has been that when using them it is difficult to separate the bacterium spores from the other components of the sediment. Furthermore, the quantitative method is exceedingly laborious.

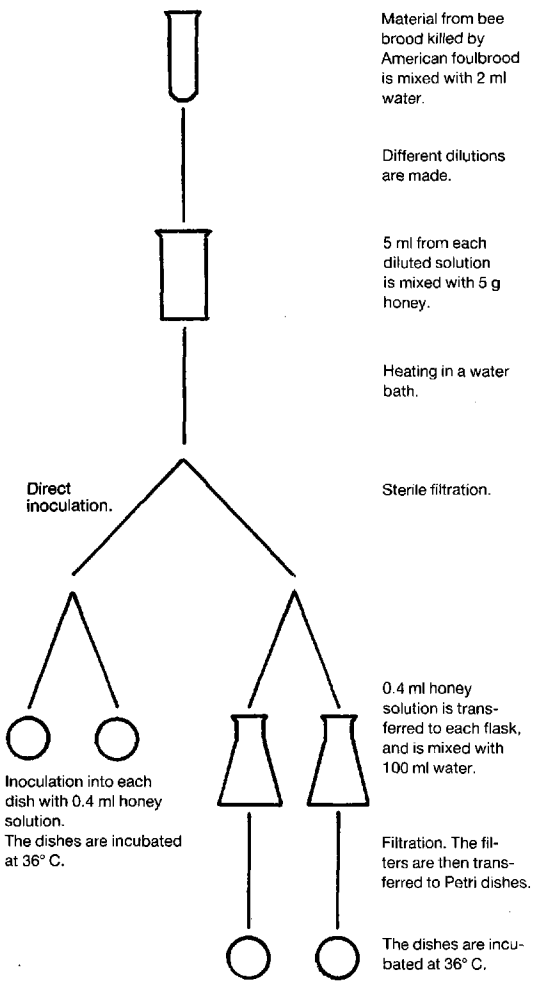

Fig. 1. Methods for determination of *B. larvae*.

In view of this, we have employed two different methods for determining the presence of *B. larvae* in honey. The present paper presents a description and comparison of these methods.

Methods

Spores of *B. larvae* were used from two comb samples where American foulbrood had killed brood of bees. The samples were removed in May–June 1979 from sick colonies of bees in two apiaries on the island of Funen. The experiments took place in the spring 1980 and the winter of 1980–81. In the period between the removal of the samples and the beginning of the experiments, and in the periods between experiments, the samples were stored in a refrigerated container at approx. –15° C. After a preliminary work, the following method was used (see Fig. 1).

A small quantity of dead brood was taken by means of an inoculation loop from each comb sample. Each piece of material was suspended in 2 ml sterile, demineralised water, and the number of spores were counted using a Thoma blood count chamber (depth of 0.05 mm). The computation was carried out following *Drews* (1976).

A number of diluted solutions were made from each spore suspension, and from each solution 5 ml was removed and mixed with 5 g honey in a beaker. The mixtures were heated in a water bath to a temperature of 88–92° C and kept at this temperature for 5 minutes. As a control, a beaker with the spore suspension from each diluted solution and a beaker with a honey solution consisting of 5 g honey and 5 ml sterile demineralised water were also taken. In addition, one other honey solution was taken to enable the temperature to be measured.

The following quantities of spores are added to the honey: 2×10^7, 2×10^6, 1×10^6, 2×10^5, 1×10^5, 5×10^4 (in six instances), 1.25×10^4 (in four instances), 5×10^3 (in two instances), 2.5×10^3 (in five instances) and 1.25×10^3.

After the period of heating, it was attempted to re-isolate *B. larvae* from the various honey solutions by means of direct inoculation of the solution and by sterile filtration.

With direct inoculation (streak culture), 0.4 ml was transferred by sterile pipette from each of the honey solutions into Petri dishes containing 10 ml of freshly prepared J-agar. Two dishes were used for each solution. The J-agar was made from 5 g tryptone, 15 g yeast extract, 3 g K_2PO_4, 20 g agar, 1000 ml demineralised water and 2 g glucose, which were separately sterilised by autoclave (*Gordon et al.*, 1973).

With sterile filtration 0.4 ml was taken from each honey solution by sterile pipette and transferred to two sterile 250 ml Erlenmeyer flasks containing 100 ml sterile demineralised water. The resulting solution was filtered through Millipore filters with a pore width of 0.22 μm. The filters were placed on Petri dishes which contained 10 ml of freshly prepared J-agar. In addition, spore suspension and honey solution from the two control beakers were inoculated into the J-agar.

With both methods, the dishes were incubated at a temperature of 36°C. Counts were performed from day 1 to day 6. Counts were carried out by determining the *B. larvae* colonies macroscopically and microscopically. For microscopy, gram stain was used, the spores being detected by means of phase contrast.

Results

When 2×10^5 to 2×10^7 *B. larvae* spores were yielded by the 5 g honey, more than 20 colonies were found on the Petri dishes. When 2.5×10^3 to 1×10^5 spores were yielded, a varying number of colonies were found on the dishes, and when there were 1.25×10^3 spores, no colonies at all were found. A table presenting the results is available from the State Bee Disease Committee on request.

Statistical analysis of the findings were undertaken. The results demonstrate that there is no significant difference between the two methods. The presence of *B. larvae* spores in honey can be determined by both methods used, when more than 10,000 spores per 5 g honey are present. Each colony found on the dishes represented 5,000–15,000 spores per 5 g honey.

Discussion

In *Sturtevant's* qualitative method for determining the presence of *B. larvae* spores i honey (*Sturtevant,* 1936), 5 cm^3 of warmed honey is dissolved in 45 cm^3 water. The solution is centrifuged twice, first for 30 minutes then for 20 minutes. After the first centrifuging most of the water is removed and fresh water added. After the second centrifuging most of the water is again removed and 0.01 ml of the sediment transferred to a slide stained with carbolic fuchsine and examined microscopically.

Sturtevant (1936) describes a quantitative method for determining the presence of *B. larvae*. With this method, 60 counts are taken of spores from the centrifuged sediment of the honey using a count chamber. The number of spores per cm^3 can then be calculated.

Sturtevant's methods are based exclusively on a morphological determination of *B. larvae* spores. The methods described in the present paper are based upon a morphological assessment of colonies, vegetative cells and spores after cultivation. The determination can, in addition, be broadened in scope to include various biochemical tests. In our opinion, using *Sturtevant's* methods, it can be problematic to distinguish *B. larvae* spores from other spores and other materials in the sediment from the centrifuged honey. Furthermore, considerable effort and energy are required to carry out the many counts needed to quantify the level of infection of *B. larvae* in the honey.

The methods employed in the present investigation also allow the level of infection to be quantified. The degree of imprecision is comparatively great, but it is probably not so great as to mean that an indication cannot be given of how great the risk is of bee brood being infected by infected honey.

Conclusion

There is no significant difference between the two methods employed in this investigation. Using these methods, the presence of *B. larvae* spores can be determined when there are more than

10,000 spores per 5 g honey. Each colony which arises after cultivation on agar represents 5,000–15,000 spores per 5 g honey. Sterile filtration demands slightly more labour than direct inoculation. The cost of materials is also rather higher with sterile filtration than with direct inoculation. Accordingly, direct inoculation seems preferable.

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