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The incidence of the foulbrood bacterium *Bacillus larvae* in honeys retailed in Denmark

Forekomst af bipestbakterien Bacillus larvae *i markedsførte honninger i Danmark*

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Summary

75 foreign and 56 Danish honeys, all of which are currently retailed in Denmark, were investigated. 81% of the foreign honeys and 23% of the Danish honeys were found to be infected with the American foulbrood bacterium *Bacillus larvae*. Half of the infected honeys (37 samples) had more than 600,000 spores per 5 g honey on average.

A method is described by which the presence of *B. larvae* can be determined in honey. The method involves the direct inoculation of honey into an artificial medium. The present method differs slightly from that described in an earlier paper.

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

Resumé

Der er undersøgt 75 udenlandske og 56 danske forskellige honninger, som er markedsført i Danmark. I 81% af de udenlandske og 23% af de danske honninger blev konstateret infektion af bipestbakterien *Bacillus larvae*. Halvdelen af de inficerede honninger (37 prøver) havde mere end gennemsnitlig 600.000 sporer pr. 5 g honning.

Der er endvidere beskrevet en metode til påvisning af *B. larvae* i honning. Ved denne metode foretages direkte podning fra honning til kunstigt substrat. Metoden er ændret lidt i forhold til en tidligere beskrevet metode.

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

Introduction

The bacterium *Bacillus larvae* is the cause of American foulbrood in the brood of honey bees. Beekeepers have often claimed that the disease is spread by retailed infected honey.

In an earlier paper (*Hansen,* 1984), two methods for determining the presence of *B. larvae* in honey were described. The first method involved the direct inoculation of the honey into a special medium. The second method involved the filtration of the honey through bacteriaproof filters, followed by culturing on the medium. No significant difference was found between the two methods, but as sterile filtration is more laborious and is somewhat more costly than direct inoculation, the first method was preferred.

In the present paper, an investigation of Danish and foreign honeys retailed in Denmark is described. The investigation was undertaken partly to test our experimental method and partly to discover to what extent retailed honey is infected with *B. larvae.*

Methods

131 different honeys were investigated, of which 56 were of Danish origin and 75 were foreign. The foreign honeys came from 22 countries. The honeys were purchased at random in different areas of Denmark in 1979, 1980 and 1981. No given batch of honey was used more than once in the investigation.

All the foreign honeys were presumed to have originated from packaging centres where several honey varieties are blended. Of the Danish honeys, 14 came from such centres, while the remaining 42 appear to have been bottled and marketed by individual beekeepers and hence to have derived solely from bee colonies in the beekeepers' own apiaries.

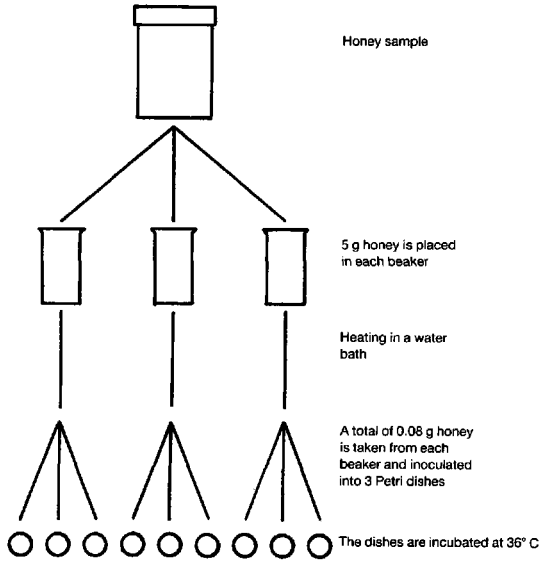

Fig. 1. Procedure followed in the investigation of honey for *B. larvae* using direct inoculation.

Direct inoculation

All samples were investigated by direct inoculation of the honey into Petri dishes containing a medium. The method is represented diagrammatically in Fig. 1. 5 g of each honey was placed in three separate sterile 50 ml beakers and these were then set in a water bath. To begin with, the temperature was maintained at 60°C for 30 minutes (effective time). However when several incubated agar dishes became overgrown with microorganisms inhibiting the growth of *B. larvae,* the conditions were modified by raising the temperature to 88–92° C for 5 minutes (effective time). Very occasionally, it was necessary to heat to 100° C for 5 minutes or more. As a control during the heating, one of the honeys already found to be infected with *B. larvae* was used.

After heating, material was taken from each beaker and inoculated into 3 Petri dishes, each of wich contained 10 ml freshly prepared J-agar. J-agar is 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 are separately sterilised and added after autoclaving (*Gordon et al.,* 1973). In an earlier investigation (*Hansen,* 1984), inoculation was carried out with 0.4 ml honey solution containing on average 0.24 g honey. With such a quantity of honey, many of the agar dishes used in that investigation became overgrown with microorganisms inhibiting the growth of *B. larvae.* The method was therefore modified so that as much honey as the opening of the inoculation loop could contain was inoculated consecutively to the 3 dishes. The total inoculated amounted on average to 0.08 g honey. The dishes were incubated at a temperature of 36° C, and counts taken on days 1 to 6 and in some instances up to day 11. Counts were obtained by macroscopic estimate of colonies (Figs. 2 and 3), by microscopy of gram stained preparations for vegetative cells using bright field (Fig. 4) and by microscopy for spores using phase contrast (Fig. 5). Our counts were performed on the basis of preliminary studies of different type cultures of certain *Bacillus*-type insect pathogens from the American Type Culture Collection (9545, 25747 and 25748 *B. larvae,*

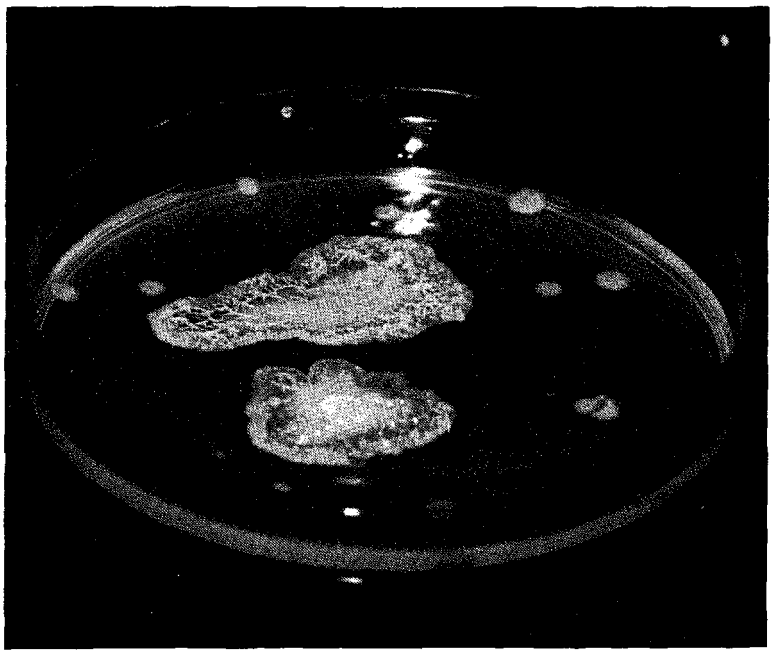

Fig. 2. Direct inoculation of honey. The small whitish colonies are *B. larvae* colonies 3 days old.

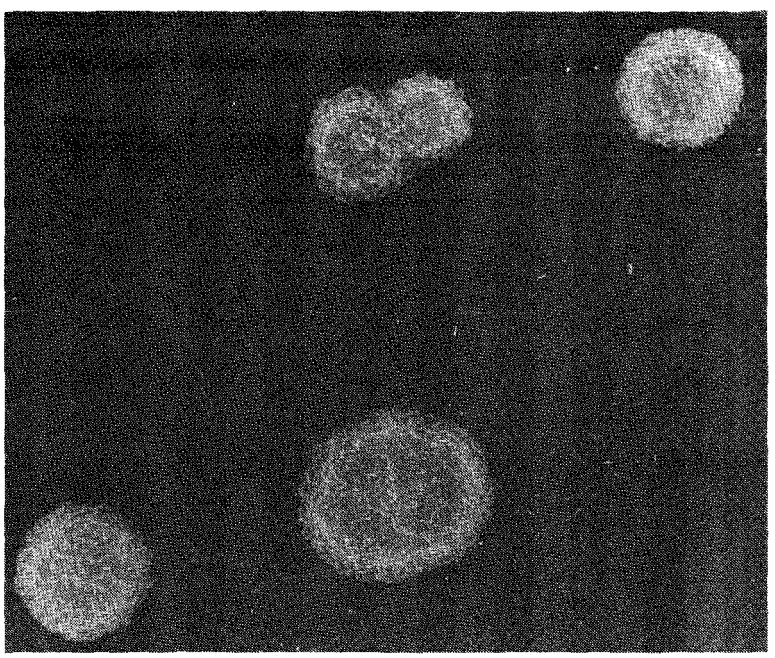

Fig. 3. The small whitish *B. larvae* colonies 3 days old from Fig. 2. The colonies are concave and have a rough surface; they can reach approx. 4–5 mm in diameter.

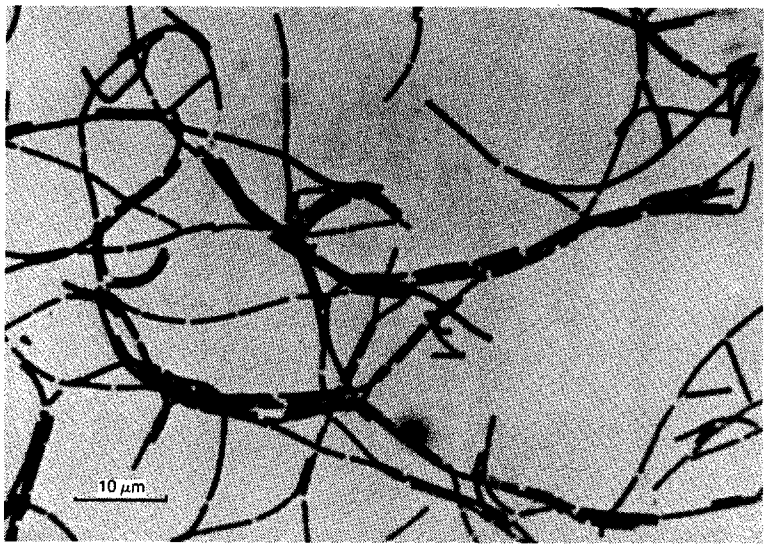

Fig. 4. *B. larvae.* Gram stained vegetative cells from colonies 2 days old. The cells reach lengths of between 2.5 and 5 μm.

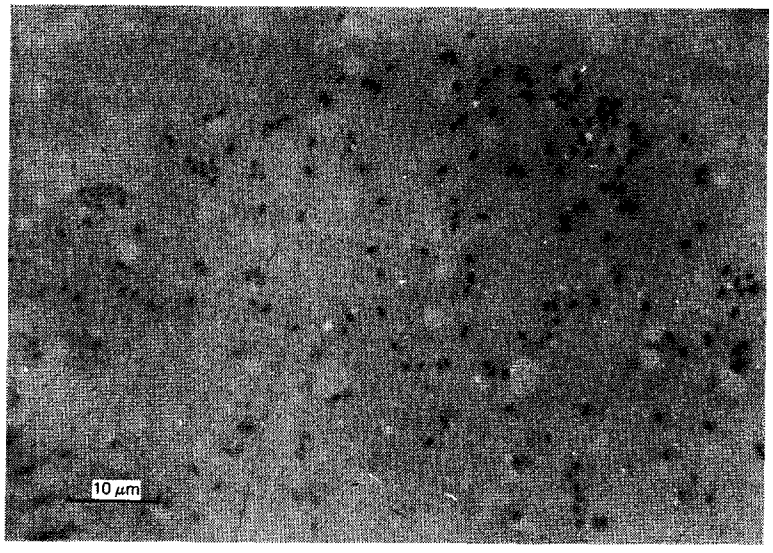

Fig. 5. *B. larvae.* Spores from colonies 9 days old (seen by means of phase contrast). The spores are approx. 1.3 × 0.6 μm.

13537 *B. pulvifaciens*, 14707 *B. lentimorbus*, and 14706 *B. popilliae*) and certain *B. larvae* isolates from Danish bee colonies with American foulbrood. In the preliminary studies, the microbiologically closely related 12980 *B. stearothermophilus*, likewise from the American Type Culture Collection, was also taken into consideration.

Sterile filtration

The honeys which were not found to be infected with *B. larvae* when the direct inoculation method was used were then investigated with Millipore filtration. This was done in order to detect the maximum number of infected honeys possible by means of the two methods developed.

1 g of each honey was taken and placed in 3 separate sterile 250 ml Erlenmeyer flasks. To facilitate temperature measurement, each flask was filled with 10 ml sterile demineralised water. There followed 5 minutes heating (effective time) in a water bath at a temperature of 88–92° C. After the period of heating, 100 ml sterile demineralised water was added to each flask. The suspension was filtered through Millipore filters with a pore width of 0.22 μm. The filters were then removed and transferred under sterile conditions to Petri dishes containing 10 ml J-agar. A control was taken of the filter and the demineralised water. As a growth control, a honey which was infected with *B. larvae* was also used. Counts were taken on days 1 to 6 using the same techniques as with direct inoculation.

Microbiological investigation

Additional, specifically microbiological investigations were also carried out with the purpose to separate *B. larvae* from other *Bacillus*-type insect pathogens and *B. stearothermophilus*. The investigations centred on catalase reaction, decomposition of casein, liquefaction of gelatin and growth in 2% NaCl. All the investigations were carried out along the lines of *Gordon et al.* (1973). In every experiment, all three *B. larvae* type cultures from the American type Culture Collection were included for comparison, and 3 dishes were used per isolate.

Results

Table 1 indicates which honeys were investigated and how many from each country were found to be infected with *B. larvae*. It emerges from the table that spores of *B. larvae* were discovered in honeys from all 23 countries that figured in the investigation.

Table 1. Number of honeys investigated and number of honeys found to be infected with *B. larvae*.

Country of origin	Total investigated	Number infected with *B. larvae*
Argentina	4	2
Australia	1	1
Bulgaria	2	1
Canada	3	2
China	2	2
Cuba	1	1
Denmark	56	13
France	5	5
Great Britain	3	2
Greece	7	6
Guatamala	1	1
Hungary	5	4
Israel	2	1
Italy	2	2
Mexico	7	6
New Zealand	2	1
Romania	1	1
Spain	3	3
Sweden	1	1
USA	1	1
USSR	2	2
W. Germany	3	1
West Indies	1	1
Foreign of unknown provenance	16	14
	131	74

In Table 2, the honeys are grouped into two sets, Danish and foreign. As the table indicates, 61 or 81% of the foreign honeys were found to be infected with *B. larvae*. 58 of these were detected by means of direct inoculation of the honey. The other 3 were revealed by means of sterile filtration, after escaping detection in the first investigation. It also emerges from the table that of the Danish honeys 13 or just under 25% were found

to be infected with *B. larvae*. All these honeys were detected by means of direct inoculation. No additional cases appeared by using sterile filtration.

Table 2. Statistics on Danish and foreign honeys.

	Number investigated	Number infected with *B. larvae*	Percentage infected honeys
Danish	56	13	23
Foreign	75	61	81
Total	131	74	56

Table 3 demonstrates that a little under 80% (11 samples) of the Danish honeys from packaging centres were found to be infected. Of the remaining Danish honeys, which were all bottled and marketed by individual beekeepers, only 5% (2 samples) proved to be infected with *B. larvae*. One of these infected honeys came from an apiary where colonies of bees had been found to be infected with American foulbrood the summer when the honey was drawn from the hives. Apparently, though, the infected honey did not actually come from the sick colonies of bees. The other batch of infected honey came from an apiary where the colonies of bees were found to be infected the year after.

Table 3. Statistics on Danish honeys.

	Number investigated	Number infected with *B. larvae*	Percentage infected honeys
From packaging centres	14	11	79
From bee-keepers	42	2	5
Total	56	13	23

In an earlier study (*Hansen*, 1984), it was demonstrated that each colony of bacteria on the Petri dishes after culturing represented 5,000–15,000 *B. larvae* spores per 5 g of the honey investigated. In the present investigation, the honey used in direct inoculation was reduced to one third of that used previously. Accordingly, each colony must now be presumed to represent 15,000–45,000 spores per 5 g honey. In Table 4, the mean total of *B. larvae* spores occuring in 5 g of the infected honeys is computed. It should be noted that some of the Petri dishes were partly overgrown with microorganisms which inhibit the growth of *B. larvae*. This being so, the spore count calculated for honeys so affected during the investigation should be taken with certain reservations.

Table 4. Degree of infection in infected honeys.

Mean total of *B. larvae* spores per 5 g honey	Less than 300,000	300,000–600,000	More than 600,000
Danish honeys:			
From packaging centres	6*	2	3
From beekeepers			2
Foreign honeys	26**	3***	32

* in 3 samples, ** in 12 samples and *** in 2 samples the Petri dishes were partly overgrown with microorganisms inhibiting growth of *B. larvae*.

Counts taken from the Petri dishes further revealed that honey bottled at packaging centres (both in Denmark and abroad) generally contained many more bacteria (apart from *B. larvae*) than Danish honeys which had been bottled and marketed by individual beekeepers. Attempts were made in a few instances to identify the bacteria involved, and in one Mexican honey *Bacillus pulvifaciens* was discovered. This bacterium is believed to be the cause of powdery scale disease which affects the brood of honey bees (*Katznelson*, 1950). This disease is not found in Danish colonies of bees.

Discussion and conclusion

Sturtevant (1932, 1936) investigated retailed honey in the USA. In all, 212 honeys were examined by him, of which 8% were infected with *B. larvae*.

No previous study of *B. larvae* in honeys retailed in Denmark has been undertaken. By means of the direct inoculation method used in the present investigation, the presence of *B. larvae* spores can be determined if there are more than 30,000 per 5 g honey. It has emerged from our experiments that in routine investigations to determine the presence of *B. larvae* it is sufficient to use macroscopic estimation of colonies of bacteria, microscopic analysis of gram stained preparations for vegetative cells using bright field, and microscopic analysis using phase contrast for spores. This being so, it should not normally be necessary to carry out any other special microbiological tests.

In our investigation we have endeavoured to include the majority of the foreign honeys retailed in Denmark at the time of experimentation, and also, as far as possible, all Danish honeys bottled at packaging centres. Even so, the total of investigated material was still comparatively limited.

Our results showed that *B. larvae* was present with equal frequency in foreign honeys and Danish honeys bottled in packaging centres. In contrast, the incidence in honeys bottled and marketed by individual beekeepers was very small. This suggests that it was just a few batches of honey containing *B. larvae* which were causing the honeys, blended and bottled at packaging centres, to be infected.

A variety of data exists on how many *B. larvae* spores are necessary to trigger American foulbrood. *Woodrow* (1942) has demonstrated that larvae under 2 days old are most receptive to the disease. *Bucher* (1958), building upon *Woodrow's* results, has shown that one half of a batch of 1 day old larvae died when they were fed 35 *B. larvae* spores. *Sturtevant* (1932) conducted a number of experiments feeding bee colonies and showed that at least 50 million spores per colony need to be present in order to American foulbrood to be triggered. Other investigations have demonstrated that as many as 10,000 million spores are required before American foulbrood appears in a colony of bees (*L'Arrivée,* 1958).

In Danish experiments with disease transmission (*Statens Bisygdomsnævn,* 1971), colonies of bees were fed with 13 different foreign honeys to investigate whether this could trigger American foulbrood. The disease broke out in colonies of bees which had been fed on 3 of the honeys. No investigation was carried out, however, to discover how many *B. larvae* spores the honeys in question contained nor whether the other honeys were infected with *B. larvae.*

The different results may be due to several factors. Different types of bees have correspondingly different degrees of inherited resistance to American foulbrood (e.g. in respect of their capacity to evacuate and the functioning of the valve flaps in the mouth of their stomachs). Furthermore, American foulbrood is a slow progressive disease, which means that a comparatively long time elapses between the time when the colony is infected and when the disease manifests itself. Doubtless, various external factors also play a role in determining how long this time span is.

In the present study, it was found that one half of the infected honeys had on average more than 600,000 spores per 5 g honey (37 samples). Not quite one half had fewer on average than 300,000 spores per 5 g honey (32 samples). If colonies of bees were to be fed on many of these honeys or were to gain access to them in some other way, then there would be some risk of American foulbrood breaking out. As we have indicated, though, how much infected honey the bees would have before becoming diseased would depend among other things upon the degree of resistance the bees possessed to American foulbrood.

It would be highly desirable if future studies could shed more light on the role played by variable inherited factors and variable external factors in outbreaks of American foulbrood within a bee colony. It would likewise be desirable to know whether American foulbrood might be reliably diagnosed in colonies of bees via investigation of their honey. If this was possible, our method could be used as a practical tool also in this respect.

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