

## **Early detection of American Foulbrood by honey and wax analysis**

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### **Summary**

The early detection of American foulbrood is possible by examination of stored food and extracted honey. Apart from the early identification of disease outbreaks, a forecast concerning the probability of an outbreak, based on the quantity of agents, is also possible. Food samples extracted from honey stored above the brood nest can be defined to individual colonies. Not only the expansion of the disease outbreak but also the verification of control measures is possible. The examination of wax on spores can be done in case of sanitation measures in an apiary and the imminent destruction of food combs. As a result, the examination of food samples on spores of *Paenibacillus larvae* larvae was adopted as a diagnostic method for the German legislation relating to infectious diseases.

### **Introduction**

The breakout of American Foulbrood (AFB) after the transfer of the pathogen agent depends, apart from the number of pathogen agents, mainly on the power of resistance of the infested bee colony. Most decisive in this respect is at which stage and to which extent the colony recognises the infected unsealed brood and removes it. AFB is normally diagnosed on the basis of their clinical symptoms. Typical for American Foulbrood is the brood being decomposed to a stringy mass. Recently, however, there have been mixed infections by viruses leading to a watery not stringy remains. To secure the diagnosis, the pathogen agent is afterwards proved in laboratory by microbiological or gene-technological methods (PCR). The diagnosis of AFB from the clinical symptoms implies the disadvantage that it can only be done after the outbreak of the disease and therefore excludes possibilities to recognise sub-clinical stages facilitating prophylaxis or controlling sanitary measures. A longer quarantine may lead to an apiculture's economy loss. An early diagnosis of AFB even in its sub-clinical stage implies therefore a most important advantage.

### **Examination of honey and stored food samples on P.I.I.**

#### **Method**

##### Extraction of honey sample and sample from stored food

- Apart from the samples taken one negative and one positive sample accompany each test series.
- The samples are heated to 34°C to facilitate an easy processing.
- 5g of the food resp. the honey sample each and 5 ml aqua dest. are homogenised in a test tube by means of a shaker.
- Each sample is tested in three parallel series.

- All samples are placed in water bath ( $90^{\circ} \pm 1^{\circ}\text{C}$ ) for 6 minutes to kill the spores of other bacteria and fungi as far as possible.
- From each test tube 80 $\mu\text{l}$  of the solution are pipetted onto three plates each (MYPGD agar or Columbia blood agar) and smeared out by a spatula. The cultures are breded in an incubator for three days at  $37^{\circ}\text{C}$ .
- The grown colonies are first differentiated from their outer characteristics. The colonies of the *Paenibacillus* larvae are white and can easily be identified by their concave form and rough surface.
- The number of colony forms with negative catalase test are determined by a culture counting apparatus..
- For definite security one colony is transferred onto Columbia slant agar and incubated for three to four days at  $37^{\circ}\text{C}$ .
- In the fluid at the bottom of the test tube plaited flagellae indicating *P.I.I.* can easily be identified, for example by means of a nigrosine preparation or on phase contrast.

The number of colonies grown by this method indicate a possible outbreak of the American Foulbrood Ohe, 1997; Ritter and Kiefer, 1995)

## Results and Discussion

In total 1520 trade honeys were examined on spores of *Paenibacillus larvae larvae*.(see fig.1). In 98% of 700 imported honeys from countries outside the EU spores could be detected. 62% of the 200 honeys evidently originating from the EU internal market contained spores. Only 2% of the domestic honeys directly commercialised by the beekeepers contained spores. The amount of spores found there was lower by the factor 100 than with the imported honeys. The control of AFB by antibiotics practised in many countries out of central Europe can be regarded as the reason for this. By this medical therapy only the vegetative form of P.I.I. is killed and not its spores. Therefore,

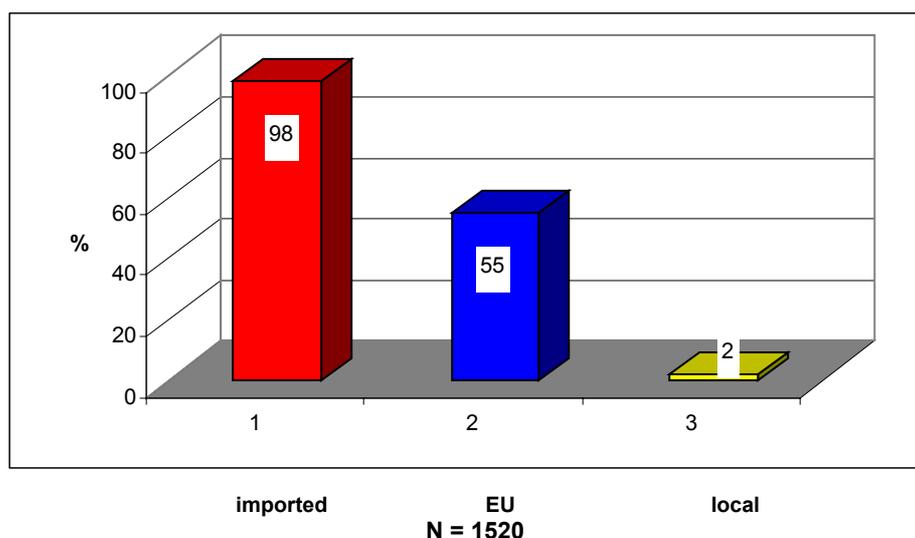


Fig.: 1 :Analysis of *P.I.I.* spores in Honey

the disease breaks out again shortly after the discontinuation of the medical treatment and consequently leads to the production of new spores. By the hygiene behaviour of the bees the food stocks are more and more contaminated. This affords a repeated treatment in short intervals.

Parallel to the examination of the domestic honeys a clinical examination of the colonies was made. In 0,4% of the apiaries, colonies with clinical symptoms of AFB could be found. Except of two apiaries, spores could already be found in the extracted honey of the remaining ones. In the other apicultures with positive honey samples as well as in apiaries with negative honey samples examined at random an outbreak could not be stated.

In order to prove the infestation rate and the infective pressure in an apiary samples of food stored near sealed brood were taken and brood cells were examined on clinical symptoms (fig. 2). On the basis of the results received the colonies could be divided into two groups: one with less and the other with more than 5000 spores on an average per g food honey. In the group with less than 5000 spores/g food honey, 2% showed clinical symptoms, in the one with more than 5000 spores/g food the rate was 88%. To profit from the results of the laboratory examination for practice, three categories of infection were chosen: Cat 0 without spores, Cat I (<5000 spores/g) and Cat II (>5000 spores/g). Cat 0 does not require any measures, Cat I demands preventive measures and Cat II a control of the probable outbreak.

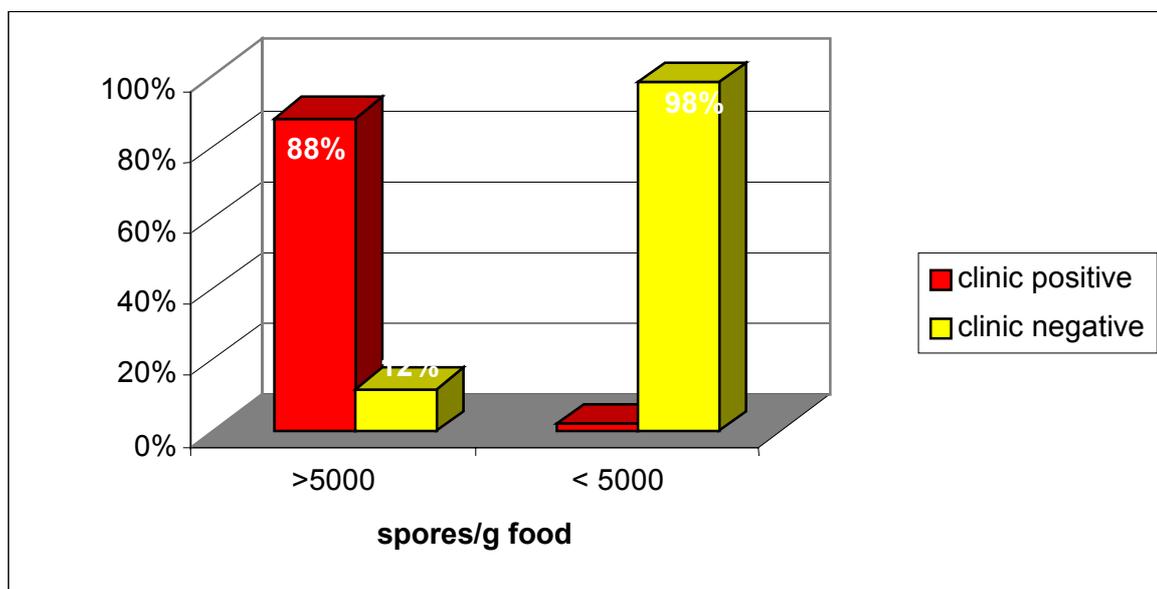


Fig.2: Contamination of food with P.I.I. and outbreak of AFB

One part of the samples was extracted in connection with outbreaks of the epidemic. With an increasing distance from the centre of the epidemic the number of spores within Cat. I found in samples of stored food decreased. Beyond a radius of 1 km, the results received the examined cases were negative. After sanitation of the infested colonies, one to two years were normally needed until no or only few spores were detected in samples of stored food of the colonies in the environment of the former centre of the epidemic. In some of the apiaries proved to be infected samples of the stored food had

already been taken, one respectively two years before the outbreak of the epidemic. In all cases the number of spores had increased within category I.. One apiary in which the infected colonies had been killed was negative during the following two years (fig. 3). Here a relapse can obviously be excluded. In another apiary, the number of spores in samples of stored food increased again continuously (fig. 4). Later it could be proved that here the focus of the epidemic had not yet been detected. But an incomplete sanitation may also lead to the same results.

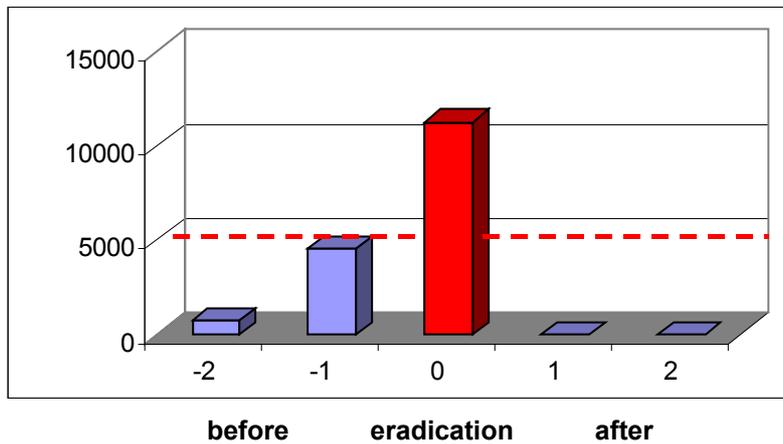


Fig.3: Contamination of food with P.I.I. spores (n spores/g) dependent on the time of outbreak (0)

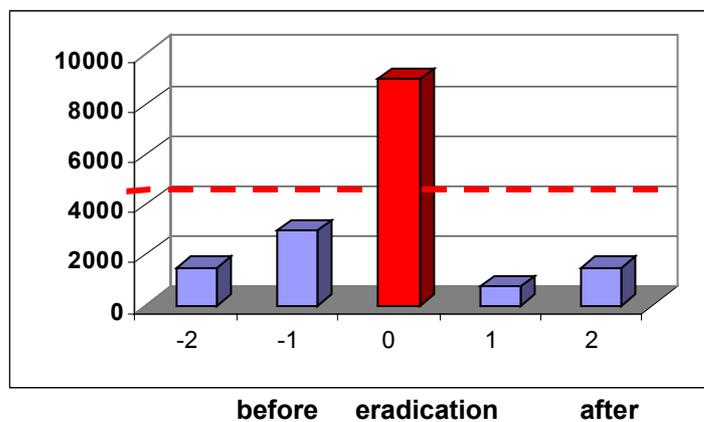


Fig. 4: Contamination of food with P.I.I. spores (n spores/g) dependent on the time of outbreak (0)

**Examination of wax samples on P.I.I.**

## Method

The method of detecting P.I.I. in honey was adapted to wax. For examination, however, the wax had to be dissolved in organic solvent (Ritter and Metzinger, 1998). Diethyl ether was the most appropriate. For detecting spores in wax, the wax was put into water (wax/water 1:10). The receptacle was placed in a water bath heated up to 90°C for 6 minutes. After cooling down the ether was added (diethyl ether/water 1:9) and then it was shaken carefully. After two minutes standing time, there was a deposit of a watery solution containing the P.I.I. spores. This was transferred to the plates as described for honey samples.

## Results and Discussion

From 420 examined comb foundations from different producers in Germany, Switzerland, Austria and Denmark 70% contained spores. In contrast to this, only 2% of 20 of the comb foundations produced by beekeepers with wax from their own wax circuit showed spores. However, the spore content in both groups was always below 1000 spores/g wax. In a field test in Denmark, the danger of infection by spores in wax was to be examined (Ritter et al, 2001). For this purpose, certain quantities of spores were introduced into the wax of midribs. In total 665 millions to 5320 millions of spores were introduced per colony. Except from few food samples three months after the start of the test, the colonies showed neither positive samples from the food wreath nor clinical symptoms during the 5 following years.

## Conclusions

Honey samples are especially suitable for a screening of AFB in a larger area. They offer the advantage that the colonies are not disturbed and an examination can be done without previous notice. The disadvantage consists in the relatively inexact results in case of bigger honey yields and low infection rates of the colonies. By means of samples from stored food the infective pressure and consequently a possible outbreak can be stated in smaller areas and individual apiaries. Another essential advantage consists in the surveillance of sanitation measures. Inadequate sanitation of an apiary as well as undetected centres of epidemics can be recognised from the increasing spore contents. Moreover, these methods enable a certified prove of AFB in bee colonies bound to be transferred to another location. In addition, the control of bee food regarding spores, required within the EU in case of imports, is facilitated. The examination of wax on spores can be done in case of sanitation measures in an apiary and the imminent destruction of food combs. This enables a selection according to contamination, at least for combs free of brood. As spores of P.I.I. remain infectious even for decades the danger of infection persists also from abandoned apiaries free of bees. It can only be excluded by means of examinations of wax samples.

## References

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