Apidologie (1992) 23, 497-501
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Original article

American foulbrood incidence in some US and Canadian honeys

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(Received 24 January 1992; accepted 5 June 1992)

Summary — Eighty-two samples of honey supplied by American and Canadian honey producers were tested by the Hansen method for the presence of American foulbrood *(Bacillus larvae)* spores. Seven (8.5%) were found to be positive. It was confirmed that the Hansen method is rapid and reliable. We believe it will be useful in screening individual colonies to determine which hives should be carefully inspected and/or treated to prevent potential economic loss from American foulbrood.

American foulbrood / *Bacillus larvae* / honey / honey bee colony / Hansen method

INTRODUCTION

American foulbrood, caused by *Bacillus larvae* White, is a serious disease of honey bees around the world resulting in considerable economic losses to beekeepers. The disease can be controlled with antibiotics but treatment costs money and is time-consuming. It would be less expensive if antibiotics could be applied only to colonies with visible signs of the disease or where the *B larvae* spore count is high. Detection of spores of *B larvae* in honey has not been easy in the past as the microorganisms are fastidious, requiring a highly nutritious growth medium (Shimanuki *et al*, 1965). Experiments by Hansen (1984a,b) have demonstrated that the relativey simple, direct inoculation of undiluted honey onto a suitable medium can be used to detect the presence of *B larvae* spores in honey. Hansen's method makes it possible, for the first time, to check honeys from individual hives for the presence of *B larvae* and to predict the desirability of using an antibiotic treatment for control in individual hives.

Sturtevant (1932, 1936) reported that a minimum of 50 000 spores/ml honey was required for detection. In his tests, 15 (8%) of 187 honey samples tested were found to be positive for *B larvae*. Shimanuki (1963) reported that < 10% of *B larvae* spores will germinate and produce visible colonies *in vitro*. Shimanuki and Knox (1988) determined that approximately 100 *B larvae* spores were required to produce growth on brain heart infusion

agar medium (at 37 °C and a 72-h incubation period). They detected *B larvae* in 100% of commercially-packed honeys, but in only 30% of beekeeper-packed honeys. Their method involved dilution of the honey, dialysis, centrifugation, resuspension, and heat-shocking at 80 °C for 10 min, followed by spreading on the agar plate. Unfortunately, they did not indicate how they identified *B larvae* colonies.

The *Australasian Beekeeper* (Editor, 1990), reported a method for the detection of *B larvae* spores that involved dilution of the honey sample with saline, centrifugation to collect the spores, and heating to 80 °C for 15 min to destroy vegetative contaminants and activate the spores. Nalidixic acid, an antibiotic, was added to the medium to suppress the growth of *B alvei.* The medium used was not specified, neither was the incubation temperature and time. The Australians tested 393 packing-plant honey samples. *B larvae* was found in 10.11% of the samples. They concluded that the procedure was an effective diagnostic tool for American foulbrood identification.

Hansen (1984a, b) developed a method in which honey was spread directly on the surface of an agar medium containing 5 g tryptone, 15 g yeast extract, 20 g agar, 3 g K_2HPO_4, and 2 g glucose/l demineralized water. The honey samples were heated to a minimum of 60 °C for 30 min, to as high as 88–92 °C for 5 min (effective time) and sometimes to 100 °C to eliminate vegetative cells. The controls were honey samples known to contain *B larvae*. Incubation was at 36 °C usually for 1 –6 (maximum of 11) days. A standard loop was used for spreading the honey over the agar plate surface. On average, it delivered 80 mg honey to the plate surface. Based on studies of honeys inoculated with known numbers of *B larvae* spores, honeys containing > 2 000 spores/g honey will yield a positive result (Hansen, 1984a). Each *B larvae* colony produced on a plate is equivalent to 1 000–3 000 spores/g honey. Triplicate plates were used per sample of honey. *B larvae* was confirmed by the production of typical colonies, by microscopic examination of stained smears to reveal typical rods, and by examination for typical spores under phase microscopy. These studies provided a method whereby large numbers of honey samples can be checked rapidly for *B larvae* spores.

Hansen (1984b) found that 74 of 131 samples (56.4%) of honeys from around the world contained *B larvae* spores; 61 of 75 (81%) foreign honeys contained *B larvae* spores; 13 of 56 (23%) of Danish honeys were positive for *B larvae.*

The above-mentioned author (Hansen, 1986) tested 532 samples of honey. Of these, 521 samples were honeys from apiaries showing no clinical symptoms of American foulbrood (AFB) either during the year the sample was collected or the following year. However, 47 samples (9.0%) contained *B larvae* spores. Eleven samples of honey were tested from apiaries showing clinical signs of AFB. Nine samples (81.8%) contained *B larvae* spores. The numbers of spores of *B larvae* triggering AFB varied. The disease was found in apiaries from which honeys in some cases contained from 6 000–12 000 spores/g. One apiary produced honey containing 3 000 000 spores/g but showed no clinical symptoms of the disease. These data suggest that there may be a strong genetic component of American foulbrood resistance. In most cases, spores of *B larvae* were found in the honey the year before an outbreak of AFB.

The purpose of our study was to test the Hansen method as an efficient, reliable method of detecting AFB spores in American honeys, mainly samples from eastern North America.

MATERIALS AND METHODS

A modified Hansen's method was used. Hansen's medium contains (per l) 20 g agar, 5 g tryptone, 15 g yeast extract, 3 g K_2HPO_4 and 2.0 g glucose. The water used in the medium was demineralized. The medium was sterilized at 121 °C for 15 min.

Approximately 20 ml of agar medium were poured into each Petri plate aseptically and allowed to set. About 80 mg of honey was streaked over as much of the agar plate surface as possible with a standardized platinum loop. The starting honey was heated to a temperature of 80 °C for 10 min prior to inoculation to destroy vegetative bacterial cells that are occasionally present and which may overgrow plates before *B larvae* spores can germinate and grow. The heating also activates the germination of *B larvae* spores. Cultures were incubated at 30 °C and counted at daily intervals for at least 6–10 days. Honey samples with a known content of *B larvae* spores were utilized as positive controls in all experiments. *B larvae* colonies were identified by typical colony form, microscopic examination of Gram-stained smears, examination of cells and spores under phase contrast microscopy, and a negative catalase reaction when flooded with 3% H_2O_2.

Eighty-two honey samples were obtained from 44 different apiaries and commercial honey producers in 12 states: New York (47), Pennsylvania (12), Massachusetts (6), Connecticut (1), New Jersey (1), Georgia (1), Ohio (6), North Carolina (1), Rhode Island (1), Virginia (1), Maryland (1), Michigan (1) and Ontario, Canada (3). Duplicate plates of Hansen's medium were streaked with single loops of honey from the various samples of honey.

RESULTS

Hansen's method proved to be reliable and easily applied. Colonies that grew in 24–48 h were not *B larvae* and consisted of environmental bacteria present in the honey. If present, *B larvae* spores germinated and colonies grew after 3–5 days. They appeared as a typical "bloom". Seven of 82 honey samples (8.5%) contained *B larvae* spores. Six of the positive samples were from New York State; one was from Pennsylvania. *B larvae* counts ranged from 10–25 colony forming units (cfu)/g honey in 3 samples, 320–440 cfu/g honey in one sample, 560 to > 3 000 cfu/g honey in 2 samples, and to 2 540 to 4 000 cfu/g honey in one sample. If one uses the probable numbers of *B larvae* spores/cfu (Hansen, 1984a,b), the actual numbers of *B larvae* spores in the honey samples ranged from 10 000 to 12 000 000/g honey (each colony representing 1 000 to 3 000 spores/g honey).

The remaining honey samples (91.5%) showed no growth of *B larvae* colonies. However, 3 samples of honey contained bacteria, not *B larvae* at levels of > 3 000 cfu/g. Five samples of honey showed no microbial growth on Hansen's medium.

CONCLUSIONS

The purpose of this study was to test the Hansen method as a reliable, efficient means of detecting AFB spores in individual honey samples. Results indicate that it is a reliable method of detecting *B larvae*. Bacteria other than *B larvae* generally grow on the inoculated plates within 2 days. *B larvae*, on the other hand, grows and appears as "bloom" of typical colonies in 3–6 days. The colonies have a characteristic appearance and when exposed to a drop of 3% H_2O_2 release no oxygen. They are catalase-negative. Microscopic examination reveals typical rods and a few spores. Thus, it is our conclusion that the Hansen method can be used to check individual hives and honey samples for the presence of *B larvae* rapidly and efficiently.

Résumé — Présence de spores de loque américaine dans certains miels des États-Unis et du Canada. On a testé

à l'aide de la méthode de Hansen la présence de spores de *Bacillus larvae* (BL), agent de la loque américaine (AFB), dans 82 échantillons de miel fournis par des producteurs de miels des USA et du Canada. Sept analyses (8,5%) se sont révélées positives. Le milieu d'Hansen a la composition suivante : pour 1 l d'eau déminéralisée 20 g de gélose, 5 g de tryptone, 15 g d'extrait de levure, 3 g de K_2HPO_4 et 2,0 g de glucose. Le milieu est stérilisé à 121 °C pendant 15 min.

Vingt ml environ du milieu gélosé sont déposés dans chaque boîte de Petri en conditions aseptiques et mis à solidifier. Le miel (environ 80 mg) est étalé sur une surface de gélose aussi grande que possible à l'aide d'un anneau en platine standardisé. Le miel a été chauffé à 80 °C avant l'inoculation afin de détruire toute cellule bactérienne éventuellement présente et susceptible de couvrir les boîtes avant que les spores de BL ne germent et croissent. Le chauffage a aussi pour action d'activer la germination des spores de BL. Les cultures sont mises à incuber à 30 °C et les comptages sont faits quotidiennement durant 6–10 j. Des échantillons de miels renfermant une quantité connue de spores de BL sont utilisés comme témoins positifs. Les colonies de BL sont identifiées d'après la forme typique de la colonie, par examen microscopique de frottis colorés par la méthode de Gram, par examen de cellules et de spores en microscopie à contraste de phases et par la réaction de catalase négative après submersion par H_2O_2 à 3%. Nos résultats prouvent qu'il s'agit d'une méthode fiable pour détecter les BL. Les bactéries autres que BL se développent généralement en 2 j dans des boîtes inoculées. BL, par contre, se développe et apparaît sous forme d'une «floraison» de colonies typiques en 3–6 j. Les colonies ont un aspect typique et, lorsqu'on les expose à une goutte de H_2O_2 à 3%, ne dégagent pas d'oxygène, prouvant ainsi qu'elles sont catalase négatives. L'examen microscopique montre des batonnets et des spores typiques. Nous en concluons que la méthode de Hansen peut être utilisée pour vérifier la présence de BL dans les colonies et les échantillons de miel avec rapidité, efficacité et à un coût relativement faible.

loque américaine / *Bacillus larvae* / miel / colonie d'abeilles / méthode Hansen

Zusammenfassung — Vorkommen von Sporen der Amerikanischen Faulbrut in einigen Honigen aus den Vereinigten Staaten und Kanada. Honigproduzenten aus den USA und Kanada stellten 82 Honigproben zur Verfügung, um sie durch die Hansen-Methode nach dem Vorkommen von Sporen der Amerikanischen Faulbrut (AFB; *Bacillus larvae*, BL) zu untersuchen. Sieben Proben (8,5%) waren positiv. Hansen's Nährmedium enthält per Liter 20 g Agar, 5 g Trypton, 15 g Hefe-Extrakt, 3 g K_2HPO_4 und 2 g Glukose. Das für das Medium benutzte Wasser war destilliert. Das Nährmedium wurde 15 min bei 121 °C sterilisiert.

Etwa 20 ml des Agarmediums wurden unter aseptischen Bedingungen je Petrischale ausgegossen und zum Erstarren gebracht. Dann wurden etwa 80 mg Honig mittels einer standardisierten Platinöse möglichst gleichmäßig über die gesamte Oberfläche der Platte ausgestrichen. Vor der Bebrütung wurden die Kulturplatten auf 80 °C erhitzt, um vegetative Bakterienzellen zu zerstören, die gelegentlich vorhanden waren und möglicherweise die Platten vor der Keimung und Vermehrung der BL-Sporen überwachsen könnten. Die Kulturen wurden bei 30 °C inkubiert und täglich für mindestens 6–10 Tage kontrolliert. Bei allen Experimenten wurden bekanntermaßen BL-Sporen enthaltende Honige als positive Kontrollen benutzt. BL-Kolonien

wurden nach der typischen Kolonieform, mikroskopischen Untersuchungen gramgefärbter Ausstriche, Untersuchung der Zellen und Sporen unter einem phasenkontrast-Mikroskop und der negativen Katalase-Reaktion nach Übergießen mit 3% H_2O_2 bestimmt.

Unsere Ergebnisse zeigen, daß dies eine verläßliche Methode zur Entdeckung von *Bacillus larvae*-Sporen ist. Andere Bakterien als BL wachsen im allgemeinen auf den beimpften Platten innerhalb von zwei Tagen. Bl hingegen erscheint als eine "Blüte" von typischen Kolonien erst innerhalb von 3–6 Tagen. Die Kolonien haben ein typisches Aussehen und sie geben nach Berührung mit H_2O_2 keinen Sauerstoff ab, womit bestätigt wird, daß sie Katalase-negativ sind. Die mikroskopische Untersuchung ergibt typische Zöpfe und typische Sporen. Wir kommen deshalb zu dem Schluß, daß die Hansen-Methode rasch, verläßlich und mit relativ geringen Kosten zur Prüfung einzelner Bienenvölker und Honigproben auf die Anwesenheit von BL eingesetzt werden kann.

Amerikanische Faulbrut / *Bacillus larvae* / Honig / Bienenvolk / Hansen Methode

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